This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

REMARKS

Claims 32-57 and 72-76 were pending in this application prior to this Response, with claims 38 and 40 being withdrawn pursuant to a restriction requirement. By the present communication, no claims are added or cancelled, and claims 32, 34, 41 and 74 have been amended to define Applicants' invention with greater particularity. The amendments add no new matter, being fully supported by the pending application and original claims. Accordingly, claims 32-57 and 72-76 are pending in this application, with claims 38 and 40 being currently withdrawn.

The Rejection under 35 U.S.C. § 112, Second Paragraph

Applicants respectfully traverse the rejection of claims 32-57 and 72-76 under 35 U.S.C. § 112, second paragraph, as being indefinite in recitation of the name "JAM" due to alleged failure to convey what the Applicants see as the invention. To overcome the rejection, Applicants have amended claim 32 to recite instead a "JAb1/Mpn domain Metalloenzyme (JAM)". In view of the amendment to claim 32, Applicants submit that all requirements under 35 U.S.C. § 112, second paragraph, are now met. Accordingly, reconsideration and withdrawal of the rejection are respectfully requested.

The Rejection under 35 U.S.C. § 103

A. Applicants respectfully traverse the rejection of claims 32-37, 39, 41, 47-57 and 72-76 under 35 U.S.C. § 103 for allegedly being unpatentable over the disclosure of Glickman et al. (*Mol and Cell Biol* 1998 18(6): 3149-3162; hereinafter "Glickman") in view of Deshaies et al. (*Annu Rev Cell Dev Biol,* 1999 15:435-67; hereinafter "Deshaies") and Reiss et al (U.S. Patent No. 5,976,796; hereinafter "Reiss"). Applicants respectfully submit that the invention methods of identifying an agent that affects isopeptidase activity, as defined by amended claim 32, distinguish over the combined disclosures of Glickman, Deshaies and Reiss by requiring: "contacting a test agent with a polypeptide comprising a JAb1/Mpn domain Metalloenzyme (JAM) domain consisting essentially of an amino acid sequence of HXHXXXXXXXXXD,

wherein H is histidine, D is aspartate, and X is any amino acid, wherein the isopeptidase activity deconjugates a modifier protein from a target protein by cleavage of a peptide bond formed between the carboxy terminus of the modifier protein and a free amino group of the target protein in proximity to a metal ion."

Applicants submit that Glickman is absolutely silent regarding a method of identifying an agent that affects isopeptidase activity of a polypeptide comprising a JAM domain consisting essentially of the claimed sequence or any other sequence. Glickman also fails to disclose that the protein subunit responsible for accomplishing the deconjugation is an isopeptidase that functions only in proximity to a metal ion, in other words, that a polypeptide comprising the JAM domain as claimed by Applicants is a novel metallo-isopeptidase. Although Glickman purports to describe the active site in the Rpn11/Mpr1 protein (Glickman page 3153 final paragraph), the description was in error, an error that those of skill in the art were quick to point out, as Applicants will detail below. Thus, Applicants submit that Glickman is silent regarding any method for identifying an agent that affects isopeptidase activity of a polypeptide that comprises a JAM domain as required by amended claim 32, and which deconjugates a modifier protein from a target protein by hydrolyzing a peptide bond "formed between the carboxy terminus of the modifier protein and a free amino group of the target protein in proximity to a metal ion."

To cure the deficiencies of Glickman for suggesting the invention methods, the Examiner relies upon Deshaies as disclosing that "ubiquitin ligases are all ubiquinated during the proteolysis in eukaryotic cytosol, wherein specific proteins are degraded by the 26S proteasome" (Office Action, page 4). However, Deshaies' disclosure is focused on the multi-enzyme systems necessary for ubiquitination of proteins, especially the SCF complex that marks a variety of regulatory proteins for destruction by the 26S proteasome. Deshaies is silent regarding the JAM domain or the exact cleavage site for which the JAM domain is active. Therefore, Applicants respectfully submit that Deshaies' comments fail to provide any suggestion as to the structure of the ubiquitin-dependent pathway, the mechanism by which a deconjugating enzyme works, or the structure of the enzymatic site in the conjugate.

Therefore, Applicants submit that the combined disclosures of Glickman and Deshaies would not motivate those of skill in the art to understand that ubiquitin is one example of a "modifier protein" whose attachment to a substrate controls cell cycling or that modification of such control can be achieved by modifying deconjugation of the "modifier protein" "via a peptide bond formed between the carboxy terminus of the modifier protein and a free amino group of the target protein". In addition, it was known in the art at the filing of the application that Ub-protein conjugates undergo two different fates: degradation of the protein substrate by the 26S proteasome *or* disassembly by ubiquitin isopeptidases to regenerate the protein substrate by disassembling the degradation signal *from only the distal end of poly-ubiquinated chains* (See attached Abstract Lam et al., "Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome" *Nature*. 1997 385(6618):737-40). Thus there is no suggestion at all in the combined disclosures of the Glickman and Deshaies that an isopeptidase having a JAM domain as disclosed by Applicants is specific for cleaving the conjugate to completely remove the modifier protein (i.e., at the peptide bond that joins the modifier protein to the target protein. Moreover, both references are silent regarding the requirement for the presence of a metal ion at the deconjugation site and regarding the presence in the metalloprotease of a JAB subunit as required by Applicants.

Moreover, Applicants respectfully submit that even if the combined disclosures of Glickman and Deshaies were sufficient to motivate those of skill in the art "to try" the invention methods there would be no reasonable expectation of success because at the time the final draft of Glickman's paper was accepted for publication, it was not yet known that the cullin subunit of SCF is modified by Nedd8. In fact, it had been incorrectly reported by the Tyers group in 1996 that cullin is modified by ubiquitin. In addition, the molecular identification of mammalian COP9 Signalosome subunits and the similarity of COP9 Signalosome to the proteasome was not reported until July of 1998. Thus, at the time of Glickman's finding, those skilled in the art could not have concluded that the COP 9/Signalosome polypeptide must be cleaving Nedd8 from cullin or deconjugating any other modifier protein from a target protein, wherein the modifier protein is conjugated to the target protein via a peptide bond formed between the carboxy

terminus of the modifier protein and a free amino group of the target protein in proximity to a metal ion.

Moreover, Glickman is in error in proposing that Rpn11 might be a cysteine-based deubiquitinating enzyme, based on a sequence alignment suggesting the existence of a conserved cysteine in a region with limited homology to other deubiquitinating enzymes. It has been shown that this alignment is bogus on theoretical grounds and in their publication Cope et al. (*Science* 298(5593):608-11, Oct. 2002, a copy of which is attached) showed that the mutation of the cysteine identified by Glickman had no effect on Nedd8 isopeptidase activity. Glickman's group reached the same conclusion and reported in a recent paper that the conserved Cys116 residue "does not appear to be an essential residue in Rpn11" (Maytal-Kivity V., et al., *BMC Biochem.* 3:28, 2002, see abstract, a copy of the reference is attached, see page 4 of 12). Thus, the structural basis for the original suggestion made in Glickman (1998) that Rpn11 is a cysteine-based deubiquitinating enzyme has been refuted by those of skill in the art.

Moreover, the cited Glickman reference suggests its own error, a suggestion completely overlooked by the Examiner in the cited reference. Glickman states: "our proteasome preparations had low activity in several deubiquitinating assays" (Glickman, p. 3158, penultimate paragraph). In the next sentence Glickman admits there is no evidence that the deubiquitinating activity shown is Rpn11-dependent. The most recent publication from Glickman's lab reaches the conclusion that the deubiquitinating activity seen in the proteasome preparation stems from Ubp6, not Rpn11. Moreover, data in R. Verma et al. unambiguously showed that Ubp6 deubiquitinating activity is irrelevant to deubiquitinating of a proteasome substrate (Science (2002) 298:611-615 and online published supplement to Verma et al., a copy of which is attached).

Accordingly, Applicants submit that those of skill in the art who read Glickman with care would not have been motivated by the combination of Glickman, Deshaies and Reiss to arrive at the invention methods. Clearly, those of skill in the art were quick to point out Glickman's errors. Even if others of skill in the art had been so motivated, Applicants submit that in view of Glickman's doubt that the deubiquitinating activity shown is actually Rpn11-dependent (referred to above), there would not have been a reasonable expectation that a broad range of biological

processes dependent upon conjugation and deconjugation of a modifier protein, such as ubiquitin or Nedd8, could be modified by utilizing an Rpn11-type molecule. Especially, there would have been no reasonable expectation that an agent could be identified that affects isopeptidase activity of a JAM-containing polypeptide by determining deconjugation of a broad range of biological processes could be modulated by contacting the peptide bond joining a modifier protein and a target protein, as required by Applicants' claims, with a polypeptide comprising a domain that consists essentially of the JAM domain required in amended claim 32 because the combined disclosures of Glickman and Deshaies fail to disclose the sequence of the active site of a JAM-containing polypeptide or the claimed substrate site for which the isopeptidase has activity.

The Examiner relies upon Reiss as disclosing that "hydroxyamates act as specific inhibitors of the binding of the NH2-terminal residue of proteins." However, Reiss actually concludes that the ubiquitin ligase has separate subsites specific for basic or bulky hydrophobic NH-2 residues for which ligation is blocked by peptide inhibitors having homologous amino acid derivatives with NH2-terminal residues similar to that of the protein.. But Reiss identifies a third type of protein that interacts with the ligase at regions of the molecule other than the NH2-terminal residue and ligation of such proteins is not blocked by peptide inhibitors having a homologous amino acid derivative with NH2—terminal residues similar to that of the protein target. Thus, while Reiss discovered that certain peptides could block *ligation* of some, but not all, protein targets, Reiss is silent regarding what blocks or promotes *cleavage*. Moreover, Reiss suggests that only peptide inhibitors can be used to block ligation of a certain segment of target molecules, leaving the question completely open as to methods for identifying non-protein based agents that modify the isopeptidase activity of polypeptides that comprise a JAM domain of the type required in Applicants' claims.

Therefore, Applicants respectfully submit that *prima facie* obviousness of the invention methods of identifying an agent that affects isopeptidase activity, as required in claim 32, is not established under 35 U.S.C. § 103 over the combined disclosures of Glickman, Deshaies, and Reiss. Accordingly, reconsideration and withdrawal of the rejection are respectfully requested.

B. Applicants respectfully traverse the rejection of claims 32 and 42-46 under 35 U.S.C. § 103(a) as being unpatentable over Glickman in view of Szalay et al. (U.S. Patent No. 5,976,796). Applicants respectfully submit that the invention methods of identifying an agent that affects isopeptidase activity, as defined by amended claim 32, distinguish over the combined disclosures of Glickman and Szalay by requiring: "contacting a test agent with a polypeptide comprising a JAb1/Mpn domain Metalloenzyme (JAM) domain consisting essentially of an amino acid sequence of HXHXXXXXXXXXXD, wherein H is histidine, D is aspartate, and X is any amino acid, wherein the isopeptidase activity-deconjugates a modifier protein from a target protein by cleavage of a peptide bond formed between the carboxy terminus of the modifier protein and a free amino group of the target protein in proximity to a metal ion."

The remarks above regarding the insufficiency of Glickman (as well as , Deshaies and Reiss) for disclosing or suggesting the invention methods, as defined by claim 32, apply equally and are incorporated here. To overcome the deficiencies of Glickman the Examiner relies upon Szalay as disclosing that "[l]uciferase and other fluorescent proteins such as green fluorescent protein are used routinely in the art to monitor protein cleavage" (Office Action, page 5). However, Applicants respectfully submit that Szalay's disclosure regarding the creation and properties of a fusion protein that combines green fluorescent protein and a luciferase are not sufficient to overcome the above enumerated deficiencies of Glickman for suggesting the invention methods of claims 32 and 42-46. Szalay is absolutely silent regarding methods for testing agents, such as small molecules, for their ability to modifying deconjugation or deubiquitination of a modifier protein and a target protein. Thus, Applicants submit that those of skill in the art would not be motivated by the combined disclosures of Glickman and Szalay to arrive at the invention methods, or if they were so motivated would lack a reasonable expectation of success in view of the confusions caused by and recognized about Glickman, as described above.

Applicants respectfully submit *prima facie* obviousness of the invention methods, as recited by claims 32-57 and 72-76, is not established by the cited art. Accordingly reconsideration and withdrawal of the rejection under 35 U.S.C. § 103(a) are respectfully requested.

In view of the above amendments and remarks, it is believed that all rejections have been overcome and passage of the claims to allowance is respectfully requested.

If the Examiner would like to discuss any of the issues raised in the Office Action, the Examiner is encouraged to call the undersigned so that a prompt disposition of this application can be achieved.

Respectfully submitted,

Date: March 4, 2004

Lisa A. Haile, J.D., Ph.D.
Registration No. 38,347
Telephone: (858) 677-1456
Facsimile: (858) 677-1465

GRAY CARY WARE & FREIDENRICH LLP
4365 Executive Drive, Suite 1100
San Diego, California 92121-2133
USPTO CUSTOMER NO. 28213

Enclosures:
Publication of Cope et al.
Publication of Maytal-Kivity et al.
Publication of Verma et al.
Abstract of Lam et al.

Role of Predicted Metalloprotease Motif of Jab1/Csn5 in Cleavage of Nedd8 from Cul1

Gregory A. Cope,[1] Greg S. B. Suh,[3]* L. Aravind,[4] Sylvia E. Schwarz,[1]† S. Lawrence Zipursky,[2,3] Eugene V. Koonin,[4] Raymond J. Deshaies[1,2]‡

COP9 signalosome (CSN) cleaves the ubiquitin-like protein Nedd8 from the Cul1 subunit of SCF ubiquitin ligases. The Jab1/MPN domain metalloenzyme (JAMM) motif in the Jab1/Csn5 subunit was found to underlie CSN's Nedd8 isopeptidase activity. JAMM is found in proteins from archaea, bacteria, and eukaryotes, including the Rpn11 subunit of the 26S proteasome. Metal chelators and point mutations within JAMM abolished CSN-dependent cleavage of Nedd8 from Cul1, yet had little effect on CSN complex assembly. Optimal SCF activity in yeast and both viability and proper photoreceptor cell (R cell) development in *Drosophila melanogaster* required an intact Csn5 JAMM domain. We propose that JAMM isopeptidases play important roles in a variety of physiological pathways.

SCF ubiquitin ligases (*1*, *2*) regulate cellular function through the ubiquitination of numerous substrates, including p27 and IκBα (*3*). SCF complexes consist of four essential subunits: Cul1, the RING-H2 protein Hrt1/Roc1/Rbx1, Skp1, and a substrate-binding F-box protein (*3*). In addition, the ubiquitin-like protein Nedd8 is conjugated to Cul1. This modification (neddylation) is essential in fission yeast (*4*) and stimulates recruitment of E2 to SCF ubiquitin ligase (*5*).

The COP9 signalosome (CSN), which is a highly conserved complex related to both the proteasome lid and the eukaryotic initiation factor 3, promotes deneddylation of Cul1 (*6*, *7*). CSN may also regulate a protein kinase that phosphorylates IκBα and p53 (*8*). CSN has been implicated in a diverse range of physiological processes, including plant and animal development (*9–11*), transcription (*12*), and signaling (*13*, *14*), but the relationship between the biochemical and physiological functions of CSN remains unclear.

Individual deletion of all known de-ubiquitinating enzymes in *Saccharomyces cerevisiae* (*15*) and mutation of a candidate active-site cysteine in the Csn5/Jab1 subunit of CSN had no effect on deneddyation of Cul1 (*15*, *16*). Thus, to elucidate the mechanism of deneddylation, we sought to identify a motif within CSN that might underlie isopeptidase activity. Iterated PSI-BLAST analysis (*17*) and sequence alignment revealed a set of eukaryotic and prokaryotic Pad1/Jab1/MPN/Mov34 domain (hereafter referred to as Jab1/MPN domain) proteins previously identified as homologs of Csn5 and Csn6 (*18–20*). Almost all prokaryotic Jab1/MPN domains possess a His-X-His-X_{10}-Asp motif (where X indicates any residue) accompanied by an upstream conserved glutamate (Fig. 1). Al-

[1]Department of Biology, California Institute of Technology (CalTech), Pasadena, CA 91125, USA. [2]Howard Hughes Medical Institute, [3]Department of Biological Chemistry, The School of Medicine, University of California at Los Angeles (UCLA), Los Angeles, CA 90095, USA. [4]National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD 20894, USA.

*Present address: Department of Biology, California Institute of Technology, Pasadena, CA 91125, USA.
†Present address: G2M Cancer Drugs AG, Frankfurt/Main, Germany.
‡To whom correspondence should be addressed. E-mail: deshaies@caltech.edu

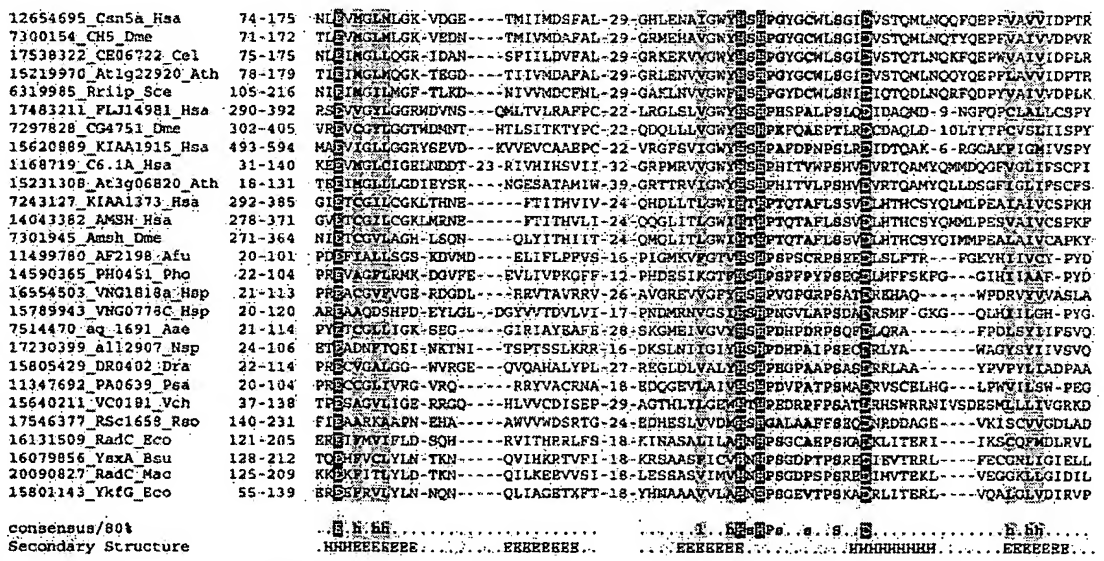


Fig. 1. Alignment of predicted JAMM domains (*34*). Selected sequences were aligned using T-Coffee (*35*) and modified manually to ensure correct superposition of the conserved motifs. A more thorough alignment is available (fig. S1). Eukaryotic proteins are grouped to reflect orthologous relationships. Position of the aligned region in the sequence is shown by numbers; poorly conserved spacers are not shown and are designated by numbers. The consensus includes amino acid residues conserved in 80% of the aligned sequences; l indicates aliphatic residues (A, I, L, V; yellow shading), h indicates hydrophobic residues (F, Y, W, A, I, L, V, M; yellow shading), and s indicates small residues (G, A, C, S, D, N, V, P; blue letters). Predicted metal-chelating and catalytic residues are shown in yellow against a dark blue background. Secondary structure prediction was made using the PHD program with the multiple alignment submitted as the query (*36*); E indicates extended conformation (β-strand), and H indicates α-helix. Each protein is denoted by the GenBank identifier followed by the gene name and abbreviated species name. Species abbreviations: Hsa, *Homo sapiens*; Dme, *Drosophila melanogaster*; Cel, *Caenorhabditis elegans*; Ath, *Arabidopsis thaliana*; Sce, *Saccharomyces cerevisiae*; Afu, *Archaeoglobus fulgidus*; Pho, *Pyrococcus horikoshii*; Hsp, *Halobacterium* sp.; Aae, *Aquifex aeolicus*; Nsp, *Nostoc* sp.; Dra, *Deinococcus radiodurans*; Psa, *Pseudomonas aeruginosa*; Vch, *Vibrio cholerae*; Rso, *Ralstonia solanacearum*; Eco, *Escherichia coli*; Bsu, *Bacillus subtilis*; Mac, *Methanosarcina acetivorans*.

though this motif is not found in all eukaryotic Jab1/MPN domain proteins (e.g., Csn6), it is conserved in a wide range of them, including all Csn5 and proteasome Rpn11 orthologs (Fig. 1). His-Asp/Glu residues often serve as ligands to immobilize a catalytic metal ion within the active site of hydrolases, including proteases (*21*). Given the exceptional conservation of residues able to chelate zinc, we hypothesized that the Csn5 JAMM motif comprises a metalloprotease that sustains Cul1 deneddylation (*22*).

To test this hypothesis, we evaluated the sensitivity of CSN-associated deneddylation activity to metal chelators (*6*). Presence of 20 mM ethylene diamine tetraacetic acid (EDTA) or 1 mM 1,10-phenanthroline (o-PT), but not 1 mM EDTA, blocked the ability of wild-type extract to restore Cul1 deneddylation activity to *csn5Δ* extract (Fig. 2A). Moreover, pretreatment of purified pig spleen CSN (*23*), but not substrate (*15*), with o-PT abolished cleavage of immunopurified Cul1-Nedd8 conjugates (Fig. 2B). Inhibition was dependent on metal chelation, because the nonchelating o-PT analog 1,7-phenanthroline had no effect on activity (Fig. 2B). These data mirror those for the LasA metalloproteases (*24*), suggesting that CSN's deneddylase activity was metal-dependent. Excess nickel added either before or after inactivation of psCSN by o-PT partially restored activity (Fig. 2B). Restoration with zinc was unsuccessful, because CSN, like the zinc metalloproteases thermolysin (*25*) and carboxypeptidase A (*26*), was inhibited by even submillimolar levels of free zinc (Fig. 2B). Importantly, o-PT had no obvious effect on the assembly of the CSN complex as judged by size exclusion chromatography (Fig. 2C). Thus, CSN-associated deneddylating activity, but not complex integrity, required metal ions.

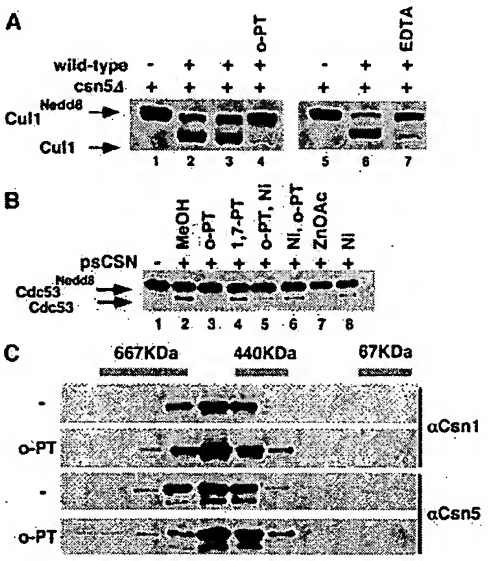


Fig. 2. CSN-dependent deneddylation of Cul1 is sensitive to divalent cation chelators. (**A**) Deneddylation of *S. pombe* Cul1 is inhibited by both o-phenanthroline (o-PT) and EDTA. *csn5Δ S. pombe* lysates (*6*) were incubated alone (lane 1 and 5) or supplemented with wild-type (Cul1-myc13) lysates (lanes 2 to 4 and 6 and 7) at 30°C for 30 min. For lanes 3, 4, and 7, both lysates were preincubated for 5 min with 0.5% methanol (MeOH) carrier, 1 mM o-PT, or 20 mM EDTA, respectively. Reactions were evaluated by western blot with antibodies to *S. pombe* Cul1 (Pcu1). (**B**) psCSN activity is modulated by metal ion concentration. psCSN was incubated for 5 min in the presence of 0.5% MeOH (lane 2), 1 mM o-PT (lanes 3 and 5), 1 mM 1,7-phenanthroline (1,7-PT, lane 4), 1 mM o-PT plus 2 mM $NiCl_2$ (Ni, lane 6), 1 mM zinc acetate (ZnOAc, lane 7), or 1 mM $NiCl_2$ (Ni, lane 8) before being mixed with immunopurified $Cul1^{Nedd8}$ (*37*). The sample in lane 5 was adjusted to 2 mM $NiCl_2$ after o-PT treatment. (**C**) o-PT does not disrupt CSN. Pig spleen CSN alone or CSN treated with 1 mM o-PT was fractionated by Superose 6 gel filtration. Fractions were analyzed by Western blotting with antibodies to either Csn1 or Csn5. Size markers are indicated above.

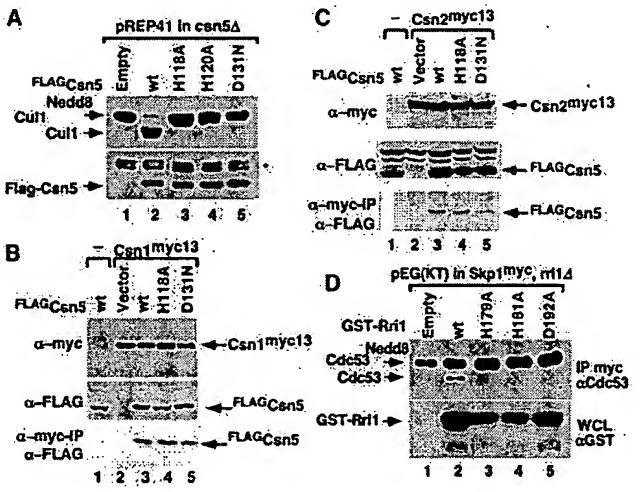


Fig. 3. Point mutations within JAMM disrupt CSN deneddylating activity but not assembly. (**A**) Mutation of either the histidine or aspartic acid residues within Csn5's JAMM motif abolishes Cul1 deneddylating activity in fission yeast. Whole-cell lysates (see SOM) from *S. pombe csn5Δ* strains carrying empty vector (lane 1) or vectors encoding ^{FLAG}Csn5(wt, lane 2), ^{FLAG}Csn5 (H118A), ^{FLAG}Csn5 (H120A), or ^{FLAG}Csn5 (D131N) were analyzed by Western blot with antibodies to *S. pombe* Cul1 (Pcu1, top panel) and FLAG (bottom panel). Asterisk denotes a nonspecific band. (**B**) and (**C**) Mutations in JAMM domain of Csn5 do not abolish CSN assembly. Extracts (*6*) from *S. pombe csn5Δ* strains carrying *csn1-myc13*$^+$ [(B), lanes 2 to 5] or *csn2-myc13*$^+$ [(C), lanes 2 to 5] and expressing FLAG-tagged wild-type or mutant Csn5 proteins were evaluated directly by Western blotting with antibodies to the Myc or FLAG tag (top and middle panels), or were first immunoprecipitated with antibodies to the Myc tag followed by Western blotting with antibodies to FLAG (bottom panel). An untagged control is shown in lane 1. (**D**) Mutations within Rri1's JAMM motif abolish Cul1 deneddylating activity in *S. cerevisiae*. *SKP1*^{Myc9}*rri1Δ* cells were transformed with the Gal-inducible vector pEG(KT) (lane 1), or the same vector encoding GST fusions to Rri1 (lane 2), Rri1(H179A) (lane 3), Rri1(H181A) (lane 4), or Rri1(D192A) (lane 5). Extracts (see SOM) were either analyzed by Western blot analysis with antibodies to GST or were first immunoprecipitated with α-myc resin followed by Western blotting with antibodies to Cdc53.

To address the function of Rri1's JAMM motif, we expressed wild-type and JAMM point mutant Rri1 proteins as glutathione *S*-transferase (GST) fusions in *rri1Δ* cells. Expression of wild-type—but not mutant—GST-Rri1 proteins partly restored deneddylated Cdc53 (Fig. 3D). Thus, despite Rri1 being the most divergent Csn5 ortholog identified to date, its JAMM motif was nevertheless required to sustain Cul1 deneddylation.

To address the physiological significance of Rri1-associated isopeptidase activity, we deleted *RRI1* in the temperature-sensitive (ts) strains *cdc53-1*, *cdc34-2*, *skp1-12*, and *cdc4-1*. Because neddylation of Cul1 enhances SCF activ-

ity (*30*), we anticipated that *rri1*Δ would suppress the ts growth of SCF mutants. Paradoxically, *rri1*Δ exacerbated both cell growth (Fig. 4A) and Sic1 turnover (Fig. 4C) defects when combined with SCF mutations.

Cosuppression of Csn5 in *Arabidopsis* partially stabilizes the SCF reporter substrate IAA6-LUC (*7*). However, it has not been established whether this effect was mediated by reduced deneddylation or loss of some other CSN activity (*8*). To test the specificity of the genetic interactions between *rri1*Δ and SCF mutants, we expressed wild-type and mutant GST-Rri1 in *cdc34-2, rri1*Δ and *skp1-12, rri1*Δ cells. Galactose (Gal)–inducible expression of wild-type GST-Rri1, but not JAMM point mutant GST-Rri1, restored growth to both *cdc34-2 rri1*Δ and *skp1-12 rri1*Δ at the restrictive temperature (Fig. 4B). Thus, the genetic interaction between SCF mutants and *rri1*Δ most likely arose from loss of JAMM-dependent isopeptidase activity. Deletion of *S. cerevisiae Nedd8* (aka *RUB1*) similarly exacerbates the ts growth defect of SCF mutants (*27*), suggesting that cycles of neddylation and deneddylation are needed to sustain optimal SCF activity.

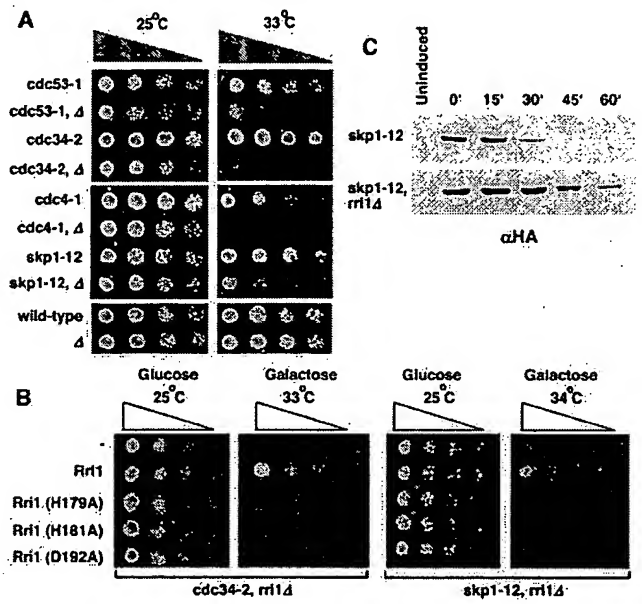


Fig. 4. Functional interaction between SCF and Rri1. (**A**) Deletion of the *RRI1* locus in *S. cerevisiae* enhances the temperature-sensitive phenotype of mutants deficient in SCF. Strains indicated were grown in complete media (YP) with dextrose (Dex) at 25°C to mid-log phase, and serial dilutions of an equal number of cells were spotted onto YP Dex plates. Cells were grown at 25°C or 33°C, and plates were analyzed after 2 to 3 days growth. (**B**) Enhancement of the temperature-sensitive phenotype of *cdc34-2 rri1*Δ and *skp1-12 rri1*Δ can be rescued by overexpression of wild-type, but not the JAMM mutant, GST-Rri1. *cdc34-2 rri1*Δ and *skp1-12 rri1*Δ strains were transformed with the Gal-inducible vectors described in Fig. 3D. Strains were grown on YP Dex at 25°C, YP Gal at 33°C for *cdc34-2 rri1*Δ, or YP Gal at 34°C for *skp1-12 rri1*Δ. (**C**) Deletion of *RRI1* in *skp1-12* impairs turnover of Sic1. *skp1-12* and *skp1-12 rri1*Δ cells carrying a *GAL-SIC1*^{HA} vector were transferred to YP Gal to express Sic1^{HA} following nocodazole-induced arrest in mitosis. A chase was then initiated by transfer of the cells to YP Dex to silence the GAL promoter, and time points were taken as indicated. Cells lysates (see SOM) were analyzed by western blot analysis with antibodies against the hemagglutinin (HA) epitope.

Whereas the lethality of *csn5*$^{Δ/Δ}$ animals was rescued by periodic heat shock–induced expression of a wild-type Csn5 transgene, no adult flies were recovered upon equivalent expression of Csn5 (D148N) (Fig. 5J). Furthermore, *CSN5*-dependent expression of the R cell markers Elav and Chaoptin was restored by expression of wild-type Csn5 (Fig. 5, G and H), but not Csn5 (D148N) (Fig. 5, E and F). These data indicate that the JAMM motif is required for an essential developmental function of Csn5 in vivo.

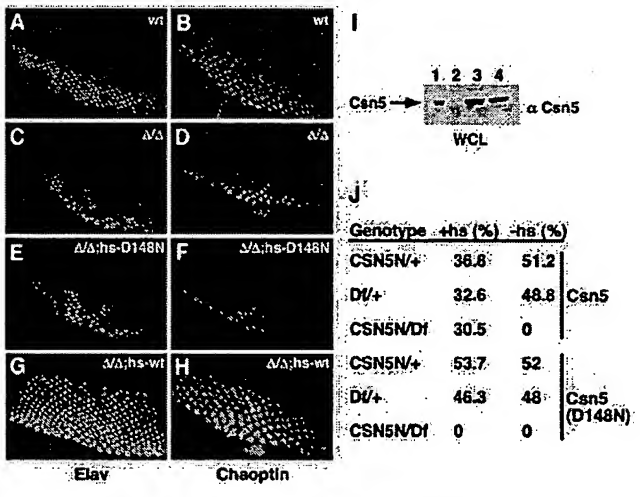


Fig. 5. Mutation of JAMM in *Drosophila* Csn5 does not rescue lethality or R cell differentiation defect in *csn5*$^{Δ/Δ}$ animals. (**A** to **H**) Developing eye disk of second/third instar wild-type [wt, (A) and (B)], *csn5*$^{Δ/Δ}$ [Δ/Δ, (C) and (D)], *csn5*$^{Δ/Δ}$;hs-Csn5(D148N) [Δ/Δ;hs-D148N, (E) and (F)], and *csn5*$^{Δ/Δ}$;hs-Csn5 [Δ/Δ; hs-wt, (G) and (H)] larvae were stained for the R cell markers ELAV [(A), (C), (E), and (G)] and chaoptin [(B), (D), (F), and (H)]. Genetics and rescue experiments, immunohistochemistry, and histology were performed as described (*11*). (**I**) Western blot analysis with anti-Csn5 antibodies (Novus) of whole-cell extracts from second/third instar larvae of wild-type (lane 1), *csn5*$^{Δ/Δ}$;hs-Csn5(D148N) (lanes 2 and 4), and *csn5*$^{Δ/Δ}$;hs-Csn5 (lane 3). For lanes 3 and 4, larvae were heat shocked at 37°C for 30 min. Larvae were subsequently harvested 60 min after heat shock and lysed as described (see SOM). (**J**) Lethality of *csn5*$^{Δ/Δ}$ cannot be rescued by JAMM mutant Csn5. Percentage recovery of genotypes (see SOM) of adult progeny obtained from crossing CSN5-null/+ to Df/+; CSN5-transgene (wild-type or D148N mutant). Percentages reflect approximately 80 adult progeny scored (see SOM). Abbreviations: CSN5N, CSN5-delete; +, wild-type CSN5; Df, deficiency-spanning CSN5 locus; hs, heat-shock (see SOM).

To begin to address whether JAMM-dependent isopeptidase activity underlies the myriad physiological functions that have been proposed for Csn5 and CSN (*8*), we tested the role of the Csn5 JAMM motif in *Drosophila melanogaster* development. Mutations in *CSN5* in *Drosophila* result in organismal lethality (*11*). At the cellular level, a range of specific defects in photoreceptor neuron differentiation have been described (*11*). Transgenic *csn5-null* (*csn5*$^{Δ/Δ}$) fly larvae carrying a P-element with wild-type (*11*) or JAMM mutant (D148N) *Drosophila CSN5* cDNAs under the control of the heat shock–dependent *hsp70* promoter were generated. Wild-type Csn5 and Csn5 (D148N) accumulated to similar levels in third instar larvae (*31*) subjected to periodic heat shocks, whereas no Csn5 protein was detected in unshocked larvae, confirming that all detectable Csn5 in these animals was transgenic (Fig. 5I). Small but equivalent fractions of wild-type Csn5 and Csn5 (D148N) molecules formed CSN complexes upon heat shock–induced expression (*15*). Inefficient assembly may be due to heat stress, because our analysis of Csn5 in *S. pombe* (Fig. 3, B and C) and Rpn11 in *S. cerevisiae* (*32*) indicated that mutation of the JAMM motif did not prevent folding of the Jab1/MPN domain.

Csn5 contains a metal-coordinating JAMM motif that was essential for cleavage of Nedd8 from Cul1. Evolutionary and structural considerations (*20*) predicted a catalytic role for JAMM, and this prediction was supported by genetic and biochemical analyses. However, we failed to detect Nedd8-directed isopeptidase with recombinant Csn5, suggesting an obligate role for complex assembly in enzyme activity. A JAMM motif in the proteasome lid component Rpn11 was likewise required for cleavage

of ubiquitin conjugates from proteasome substrates (*32*). There exist other Csn5/Rpn11 homologs in eukaryotes (Fig. 1) and, by extension, we propose the "JAMMIN" hypothesis, which posits that eukaryotic JAMM proteins are isopeptidases that deconjugate Nedd8 or other ubiquitin-like proteins.

Drosophila sustained by Csn5 carrying a JAMM domain mutation arrest development as larvae with abnormalities in photoreceptor differentiation, suggesting that at least two functions associated with Csn5—viability and photoreceptor differentiation—require its JAMM-dependent isopeptidase activity. Given that Csn5 has been implicated in c-jun signaling (*12*), p27 turnover (*33*), cytokine signaling (*14*), and growth cone-target interactions (*11*), it will be interesting to see if isopeptidase activity of Csn5 underlies these diverse processes as well.

All neddylated proteins known are members of the cullin family. It is not clear whether CSN isopeptidase acts exclusively upon cullin-Nedd8 conjugates or cleaves other targets. Regardless, given the large number of F-box proteins and the potential for substantial diversity in the substrates for SCF and other cullin-based ubiquitin ligases, CSN deneddylase activity may play an enormous role in cellular regulation.

References and Notes

1. D. Skowyra, K. L. Craig, M. Tyers, S. J. Elledge, J. W. Harper, *Cell* **91**, 209 (1997).
2. R. M. Feldman, C. C. Correll, K. B. Kaplan, R. J. Deshaies, *Cell* **91**, 221 (1997).
3. R. J. Deshaies, *Annu. Rev. Cell Dev. Biol.* **15**, 435 (1999).
4. F. Osaka *et al.*, *EMBO J.* **19**, 3475 (2000).
5. T. Kawakami *et al.*, *EMBO J.* **20**, 4003 (2001).
6. S. Lyapina *et al.*, *Science* **292**, 1382 (2001).
7. C. Schwechheimer *et al.*, *Science* **292**, 1379 (2001).
8. M. Seeger, C. Gordon, W. Dubiel, *Curr. Biol.* **11**, R643 (2001).
9. N. Wei, X. W. Deng, *Trends Genet.* **15**, 98 (1999).
10. S. Freilich *et al.*, *Curr. Biol.* **9**, 1187 (1999).
11. G. S. Suh *et al.*, *Neuron* **33**, 35 (2002).
12. F. X. Claret, M. Hibi, S. Dhut, T. Toda, M. Karin, *Nature* **383**, 453 (1996).
13. R. Kleemann *et al.*, *Nature* **408**, 211 (2000).
14. E. Bianchi *et al.*, *Nature* **404**, 617 (2000).
15. G. Cope, R. Deshaies, unpublished data.
16. C. Zhou *et al.*, *BMC Biochem.* [online] **2**, article #7 (2001). Available at: www.biomedcentral.com/bmcbiochem.
17. S. F. Altschul *et al.*, *Nucleic Acids Res.* **25**, 3389 (1997).
18. L. Aravind, C. P. Ponting, *Protein Sci.* **7**, 1250 (1998).
19. K. E. Mundt *et al.*, *Curr. Biol.* **9**, 1427 (1999).
20. C. P. Ponting, L. Aravind, J. Schultz, P. Bork, E. V. Koonin, *J. Mol. Biol.* **289**, 729 (1999).
21. N. D. Rawlings, A. J. Barrett, *Methods Enzymol.* **248**, 183 (1995).
22. Like JAMM, metal-dependent peptidases often possess two conserved acidic residues: one that acts as a ligand for zinc and a second that acts as a nucleophile to promote attack by water on the peptide backbone. Based on the common occurrence of Glu as a nucleophile and on the predicted secondary structure of the Jab/MPN domain, we propose that the Glu helps polarize a water molecule for hydrolytic attack and the COOH-terminal His and Asp residues serve as three ligands for zinc.
23. N. Wei, X. W. Deng, *Photochem. Photobiol.* **68**, 237 (1998).
24. A. G. Loewy *et al.*, *J. Biol. Chem.* **268**, 9071 (1993).
25. D. R. Holland, A. C. Hausrath, D. Juers, B. W. Matthews, *Protein Sci.* **4**, 1955 (1995).
26. M. Gomez-Ortiz, F. X. Gomis-Ruth, R. Huber, F. X. Aviles, *FEBS Lett.* **400**, 336 (1997).
27. D. Lammer *et al.*, *Genes Dev.* **12**, 914 (1998).
28. D. Liakopoulos, G. Doenges, K. Matuschewski, S. Jentsch, *EMBO J.* **17**, 2208 (1998).
29. A. C. Gavin *et al.*, *Nature* **415**, 141 (2002).
30. V. N. Podust *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 4579 (2000).
31. Null mutant survival up to third instar larvae was context dependent. Although Csn5-null larvae rarely survived to third instar at UCLA, we routinely found that these mutants survived to third instar at CalTech.
32. R. Verma *et al.*, *Science* **298**, 611 (2002).
33. K. Tomoda, Y. Kubota, J. Kato, *Nature* **398**, 160 (1999).
34. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
35. C. Notredame, D. G. Higgins, J. Heringa, *J. Mol. Biol.* **302**, 205 (2000).
36. B. Rost, C. Sander, R. Schneider, *Comput. Appl. Biosci.* **10**, 53 (1994).
37. Materials and Methods are available as supporting material on *Science* Online.

38. We thank D. Wolf, M. Hochstrasser, K. Mundt, and A. Carr for generously providing yeast strains and plasmids, the S. Benzer lab for providing lab equipment and space, and S. Lyapina for psCSN. We thank members of the Deshaies lab for providing helpful insight and discussions. This work was supported by NIH (G.A.C.) and the Howard Hughes Medical Institute.

Supporting Online Material

www.sciencemag.org/cgi/content/full/1075901/DC1
Materials and Methods
Figs. S1 and S2
References and Notes

9 July 2002; accepted 7 August 2002
Published online 15 August 2002;
10.1126/science.1075901
Include this information when citing this paper.

Role of Rpn11 Metalloprotease in Deubiquitination and Degradation by the 26*S* Proteasome

Rati Verma,[1] L. Aravind,[2] Robert Oania,[1] W. Hayes McDonald,[3] John R. Yates III,[3] Eugene V. Koonin,[2] Raymond J. Deshaies[1]*

The 26*S* proteasome mediates degradation of ubiquitin-conjugated proteins. Although ubiquitin is recycled from proteasome substrates, the molecular basis of deubiquitination at the proteasome and its relation to substrate degradation remain unknown. The Rpn11 subunit of the proteasome lid subcomplex contains a highly conserved Jab1/MPN domain–associated metalloisopeptidase (JAMM) motif—$EX_nHXHX_{10}D$. Mutation of the predicted active-site histidines to alanine (*rpn11AXA*) was lethal and stabilized ubiquitin pathway substrates in yeast. $Rpn11^{AXA}$ mutant proteasomes assembled normally but failed to either deubiquitinate or degrade ubiquitinated Sic1 in vitro. Our findings reveal an unexpected coupling between substrate deubiquitination and degradation and suggest a unifying rationale for the presence of the lid in eukaryotic proteasomes.

Proteolysis by the 26*S* proteasome proceeds by binding of the ubiquitinated substrate protein to the 19*S* regulatory particle, followed by its unfolding and translocation into the lumen of the 20*S* core, where it is degraded by the action of the 20*S* peptidases (*1*–*3*). At some point in this process, the ubiquitin targeting signal is detached from the substrate. It is appealing to envision that this deubiquitination is obligatorily coupled to degradation. Such coupling would render the targeting event irreversible, prevent unproductive turnover of ubiquitin, and presumably alleviate steric blockade of the 20*S* core entry portal by the bulky ubiquitin chain, which is linked by isopeptide bonds. When and where substrate deubiquitination takes place, the identity of the deubiquitinating enzyme (DUB), and whether deubiquitination of a substrate is essential for its degradation by the proteasome are unclear (*4*, *5*).

Budding yeast ubiquitinated S-Cdk inhibitor Sic1 (Ub-Sic1) is rapidly degraded by purified 26*S* proteasomes (*3*, *6*) in a reaction that recapitulates physiological requirements for Sic1 proteolysis (*7*, *8*). To investigate whether degradation of Sic1 is normally accompanied by its deubiquitination, we evaluated the fate of Ub-Sic1 after inhibition of 26*S* proteolytic activity. Epoxomicin inhibits the proteasome by covalently binding the catalytically active β subunits of the 20*S* core (*9*). Purified 26*S* proteasomes were preincu-

[1]Department of Biology and Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA 91125, USA. [2]National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD 20894, USA. [3]Department of Cell Biology, The Scripps Research Institute, San Diego, CA 92037, USA.

*To whom correspondence should be addressed. E-mail: deshaies@caltech.edu

BMC Biochemistry

BioMed Central

Research article

Open Access

MPN+, a putative catalytic motif found in a subset of MPN domain proteins from eukaryotes and prokaryotes, is critical for Rpn11 function

Vered Maytal-Kivity[1], Noa Reis[1], Kay Hofmann[2] and Michael H Glickman*[1]

Address: [1]Dept. of Biology and Institute for Catalysis Science and Technology (ICST) Technion – Israel Institute of Technology, Israel and [2]Bioinformatics Group, MEMOREC Stoffel GmbH, Germany

E-mail: Vered Maytal-Kivity - veredm@tx.technion.ac.il; Noa Reis - noareis@tx.technion.ac.il; Kay Hofmann - kay.hofmann@memorec.com; Michael H Glickman* - glickman@tx.technion.ac.il

*Corresponding author

Published: 20 September 2002

BMC Biochemistry 2002, 3:28

Received: 24 July 2002
Accepted: 20 September 2002

This article is available from: http://www.biomedcentral.com/1471-2091/3/28

© 2002 Maytal-Kivity et al; licensee BioMed Central Ltd. This article is published in Open Access: verbatim copying and redistribution of this article are permitted in all media for any purpose, provided this notice is preserved along with the article's original URL.

Abstract

Background: Three macromolecular assemblages, the lid complex of the proteasome, the COP9-Signalosome (CSN) and the eIF3 complex, all consist of multiple proteins harboring MPN and PCI domains. Up to now, no specific function for any of these proteins has been defined, nor has the importance of these motifs been elucidated. In particular Rpn11, a lid subunit, serves as the paradigm for MPN-containing proteins as it is highly conserved and important for proteasome function.

Results: We have identified a sequence motif, termed the MPN+ motif, which is highly conserved in a subset of MPN domain proteins such as Rpn11 and Csn5/Jab1, but is not present outside of this subfamily. The MPN+ motif consists of five polar residues that resemble the active site residues of hydrolytic enzyme classes, particularly that of metalloproteases. By using site-directed mutagenesis, we show that the MPN+ residues are important for the function of Rpn11, while a highly conserved Cys residue outside of the MPN+ motif is not essential. Single amino acid substitutions in MPN+ residues all show similar phenotypes, including slow growth, sensitivity to temperature and amino acid analogs, and general proteasome-dependent proteolysis defects.

Conclusions: The MPN+ motif is abundant in certain MPN-domain proteins, including newly identified proteins of eukaryotes, bacteria and archaea thought to act outside of the traditional large PCI/MPN complexes. The putative catalytic nature of the MPN+ motif makes it a good candidate for a pivotal enzymatic function, possibly a proteasome-associated deubiquitinating activity and a CSN-associated Nedd8/Rub1-removing activity.

Background

Many regulatory proteins are removed from the cell in a timely and specific manner by a large multi subunit enzyme called the proteasome [1,2]. For proteins to be recognized by the proteasome, they are usually first covalently attached to a polyubiquitin chain via a cascade of ubiquitinating enzymes. This ubiquitination process is reversible. Specific cysteine proteases known as DUBs (deubiquitinating enzymes) can hydrolyze the amide bond between the Carboxy-terminus of ubiquitin and an

amino group on the substrate [3,4]. Proteolysis takes place within the 20S core particle (CP) of the proteasome, while the 19S regulatory particle (RP) binds polyubiquitinated substrates, unfolds, and translocates them into the 20S CP for proteolysis. The discovery that the 19S regulatory particle of the proteasome (RP) can be separated into two discrete subcomplexes, the lid and the base, suggests that they have distinct roles in preparing a substrate for degradation [5]. The base contains six ATPase subunits, Rpt1-6, as well as the two largest non-ATPase subunits Rpn1 and Rpn2, and plays a role in anchoring the substrate, unfolding it and gating the channel leading into the 20S CP [6–9]. The lid complex consists of eight non-ATPase subunits whose functions have not been defined. All subunits of the lid subcomplex contain one of two structural motifs: six contain a PCI domain (Proteasome, COP9, eIF3), while the other two (Rpn8 and Rpn11) contain an MPN domain (Mpr1, Pad1 N-terminal) [5,10,11]. These domains are found in members of two other eukaryotic macromolecular assemblages as well: the COP9 signalosome (CSN) and the eukaryotic translation initiation factor 3 (eIF3). The functions of these domains are not known, but they are necessary for proper interactions between subunits of these complexes [12,13]. The lid appears to be required for the degradation of polyubiquitinated substrates but not for hydrolysis of unstructured or short polypeptides [5]. Thus, one possibility is that the lid is required in one way or another for proper interactions with polyubiquitinated chains.

At 66% identity between the human and yeast forms, the MPN domain protein Rpn11 is the most highly conserved non-ATPase subunit of the 19S RP, on par only with the highly conserved ATPase subunits, suggesting that it too may play an enzymatic role within the RP [14,15]. Mutations in *RPN11* cause cell cycle and mitochondrial defects, temperature sensitivity, and sensitivity to DNA damaging reagents such as UV or MMS, underscoring the importance of this subunit in proteasome function [16–18]. Rpn11 is one of a minority of proteasome subunits that exhibit dominant phenotypes upon overexpression. High dosage of human or *S. pombe* Rpn11 orthologs confer multidrug and UV resistance [19,20]. These effects may be linked to the stabilization of c-Jun observed upon overexpression of the Rpn11 subunit in *Schistosoma*, SmPOH1 [21]. In another case, however, overexpression of Rpn11 can suppress an *srp1* mutation, the yeast homolog of importin alpha, and enhance degradation of a proteasome substrate [22], illustrating that effects of Rpn11 are pleiotropic. Together, these results may suggest that Rpn11 embodies an intrinsic enzymatic activity.

In order to gain insight into the functions and evolutionary history of MPN domain proteins such as Rpn11, we performed extended database searches using the generalized profile method [23]. An improved MPN domain profile detected a large number of novel significant matches including some prokaryotic proteins from archaeal and eubacterial origins, which until now have not been known to contain MPN domains. In addition to being structurally related to the published eukaryotic MPN domains, all prokaryotic MPN domains contain an additional pattern of five polar amino acids, which are conserved in a highly correlated fashion. This motif is embedded within the conventional MPN domain, and is also conserved in some – but not all – eukaryotic MPN proteins. We therefore term it the MPN+ motif. The polar nature and coordinated conservation of the MPN+ residues suggest a catalytic and/or metal-binding function. Since Rpn11 is one of the eukaryotic MPN domain proteins harboring the MPN+ motif, we used mutational analysis to assess the importance of these conserved amino acids for the function of Rpn11 in *S. cerevisiae*. Rpn11 also contains a highly conserved cysteine residue that is not part of the MPN+ motif but is present in a number of its close paralogs such as the COP9 signalosome subunit, Csn5. It has been suggested that this may correspond to the active site cysteine of a catalytic DUB motif [14]. Therefore, we also analyzed mutants of Cys116, and compared them to mutations in the MPN+ residues of Rpn11.

Results

Extending the scope of the MPN domain

In order to identify distantly related members of the MPN domain family, we constructed generalized profiles of previously established MPN proteins. Included in the profile construction were the proteasome lid components Rpn8 and Rpn11, the COP9 signalosome components Csn5 and Csn6, and the translation initiation factor 3 components eIF3f and eIF3h, all from various eukaryotic species. After scaling of the profile [24], significant hits were found in protein database, including the STAM-interacting protein AMSH [25], and the uncharacterized human proteins C6.1A and KIAA1915. Subsequently, iterative profile refinement [24] was used to make the profile searches more sensitive. For that purpose, the newly identified MPN proteins were included in the profile construction process. After four iteration cycles, a stable set of significantly matching *bona fide* MPN proteins was identified. Representative members of this superfamily are shown in Figure 1.

In addition to the previously known MPN proteins, a number of prokaryotic members were identified, including the phage tail assembly protein K from the bacteriophage lambda and its closely related homologs from other phages and prophages. Even archaeal members of the MPN domain family were found, including predicted proteins from *Archaeoglobus fulgidus*, *Methanobacter thermoautotrophicum* and from various *Pyrococcus* species (Fig.

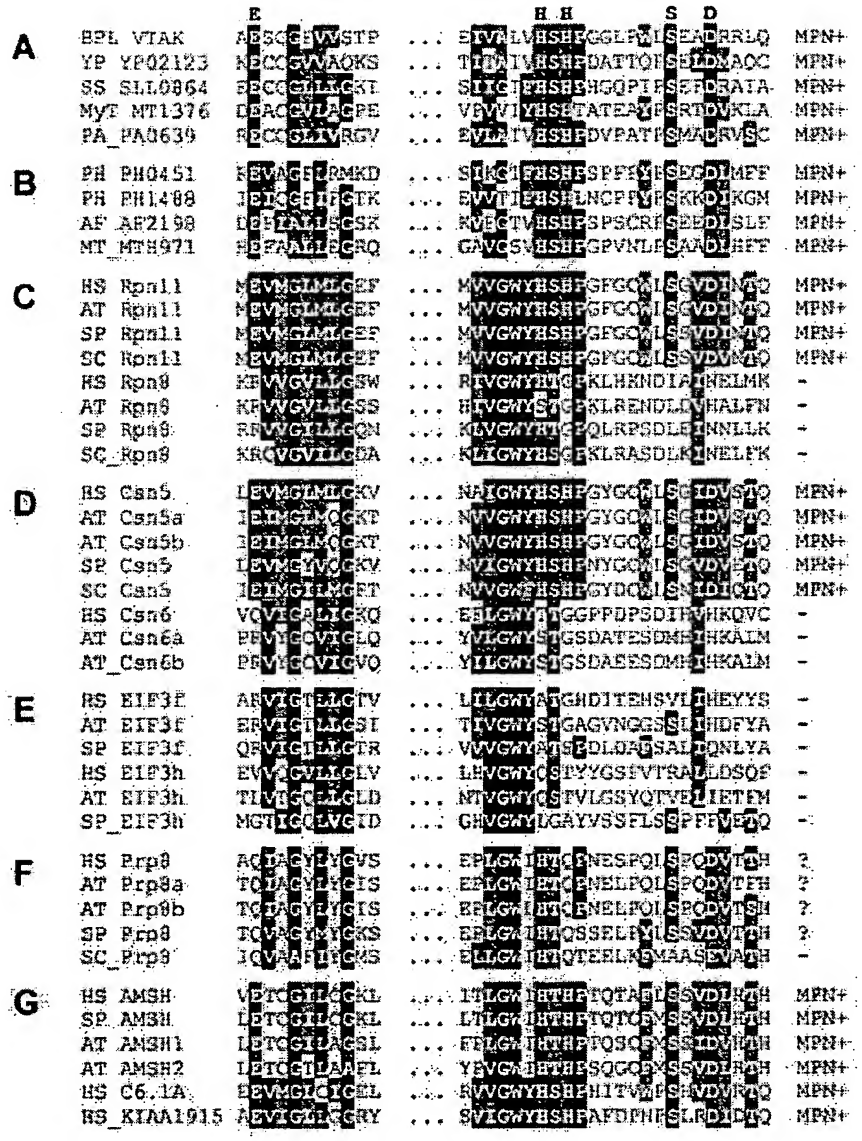


Figure 1

The MPN+ motif. Sequence alignment of representative MPN domain protein, only two conserved blocks containing the MPN+ motif are shown. The proteins are grouped in seven categories: A, bacterial; B, archaeal; C, proteasome lid components; D, CSN components; E, eIF3 components; F, Prp8-like proteins; G other eukaryotic MPN proteins. In the eukaryotic groups, representative sequences from human (HS), Arabidopsis (AT), *S. pombe* (SP) and *S. cerevisiae* (SC) are shown. Prokaryotic species shown are lambda phage (BPL), *Yersinia pestis* (YP), *Synechocystis sp.* (SS), *Mycobacterium tuberculosum* (MyT), *Pseudomonas aeruginosa* (PA), *Pyrococcus horikoshii* (PH), *Archaeoglobus fulgidus* (AF), *Methanobacter thermoautotrophicum* (MT). Residues invariant or conservatively substituted in at least 50% of all sequences are shown on black and grey background, respectively. The MPN+ motif residues are shown in red. The top line indicates the amino acids constituting the MPN+ motif; the rightmost column indicates whether a protein is considered an MPN+ protein or a plain MPN protein.

1). These proteins are the shortest MPN protein identified so far and most likely correspond to the structural core region of the domain. Since the finding of prokaryotic MPN proteins was highly unexpected, the validity of the assignment was confirmed by profile searches starting from the bacteriophage proteins that resulted in the same stable set of significantly matching proteins.

Definition of the MPN+ motif

Overall, there are no residues that are invariant throughout the MPN domain superfamily. However, while analyzing the alignment of the newly identified proteins, it became apparent that there are a number of polar residues that are conserved in a highly coordinated fashion in a subset of MPN domains (highlighted in Red in Fig. 1). These amino acids form a pattern, referred to herein as the 'MPN+ motif', which is part of the conventional MPN domain and embedded within it. The MPN+ motif contains a well-defined pattern of 'H-x-H-x[7]-S-x[2]-D', where x[7] and x[2] indicate stretches of seven and two non-conserved residues, respectively. In addition to this conserved arrangement of four polar residues, there is an additional glutamate residue in a more N-terminal region of the domain, whose conservation is perfectly correlated with the occurrence of the motif (Figure 1). An aromatic residue, preferentially a tryptophane, is found two positions upstream of the conserved serine in most but not all MPN+ proteins. Thus, it should not be considered a part of the core MPN+ motif.

With the possible exception of Prp8, all MPN domain proteins shown in Figure 1 can unambiguously be classified as either belonging to the MPN+ or to the 'plain' MPN class. The validity of this distinction is underscored by the fact that invariably all observed orthologs of MPN+ proteins also belong to the MPN+ class. For instance, the proteasome lid complex from all eukaryotes contains one MPN+ protein (Rpn11/S13) and one plain MPN protein (Rpn8/S12). The same is true for the analogous CSN complex in multicellular eukaryotes, where the MPN+ protein is Csn5 and the plain MPN protein is Csn6. In fission yeast, Csn6 appears to be absent. The same is true for the recently identified CSN-like complex of budding yeast; it contains only Csn5 but not Csn6 (Hofmann and Glickman; submitted for publication). The two MPN proteins of the eIF3 complex are both of the plain MPN type in multicellular eukaryotes and fission yeast, while in budding yeast they appear to be missing altogether. Notably, all newly identified prokaryotic MPN proteins are of the MPN+ type; the same is true for the remaining unassigned 'orphaned' MPN proteins in eukaryotes (Fig. 1). It should be stressed that the MPN+ motif is not a stand-alone motif to be found independently of the MPN domain; thus, all MPN proteins can be classified as either MPN+ or plain MPN proteins.

Mutants in the MPN+ motif of Rpn11 exhibit severe growth defects

In order to assess the importance of the MPN+ residues for efficient Rpn11 function, we performed site-directed mutagenesis of His111, Ser119, Asp122, which are part of the conserved HxH-S-D sequence that defines the MPN+ motif. In addition, Cys116, which is not part of the MPN+ motif but is highly conserved between Rpn11 and Csn5 orthologs might be a candidate for an active site residue, was mutagenized too. As seen in Fig. 2, alanine substitutions in the MPN+ motif of Rpn11 cause severe growth defects, temperature sensitivity, and sensitivity to amino acid analogs such as canavanine. Substitution mutations at different locations in the MPN+ motif display similar, but slightly distinct phenotypes. Specifically, the His111Ala mutant is extremely slow growing at 25°C and lethal when plated at elevated temperatures, even as low as 30°C. The Asp122Ala and Ser119Ala mutants are viable but slow growing at 25°C and 30°C, and lethal when shifted to 37°C or when exposed to amino acid analogs. By contrast, substitutions of the conserved Cys at position 116 showed no growth defect, and the cells appeared normal under all conditions tested.

Stress conditions such as exposure to elevated temperature or amino acid analogs are known to promote accumulation of damaged proteins, which must be removed by the proteasome [26]. Since defects in the MPN+ motif of Rpn11 cause heightened sensitivity to such conditions, it is reasonable to assume that proteasome function is jeopardized in these mutants. We conclude that the MPN+ motif is critical for the proper function of Rpn11 within the proteasome, but that Cys116 does not appear to be an essential residue in Rpn11.

Multiubiquitinated proteins accumulate in MPN+ motif mutants

In order to test whether proteasome function is indeed hampered in these rpn11 mutants, we checked the effects of mutations on the ubiquitination pattern of cellular proteins. Whole cell extracts from rapidly growing WT or MPN+ motif mutants were separated by SDS PAGE and immunoblotted with anti-Ub antibody (Fig. 3). High molecular weight polyubiquitin-conjugates are not detected at appreciable levels in extracts from yeast containing normal proteasomes since they are rapidly turned over. High molecular weight polyubiquitinated proteins do accumulate, however, in *rpn11* MPN+ mutants, indicating defective proteasome activity. In this assay, all mutations in MPN+ residues behave similarly. His111 mutants also accumulate polyubiquitinated proteins when grown at 25°C, but since this strain is extremely slow growing it was difficult to prepare extracts for comparison (not shown). No such accumulation is seen in cells containing Rpn11 with Cys116 substitutions.

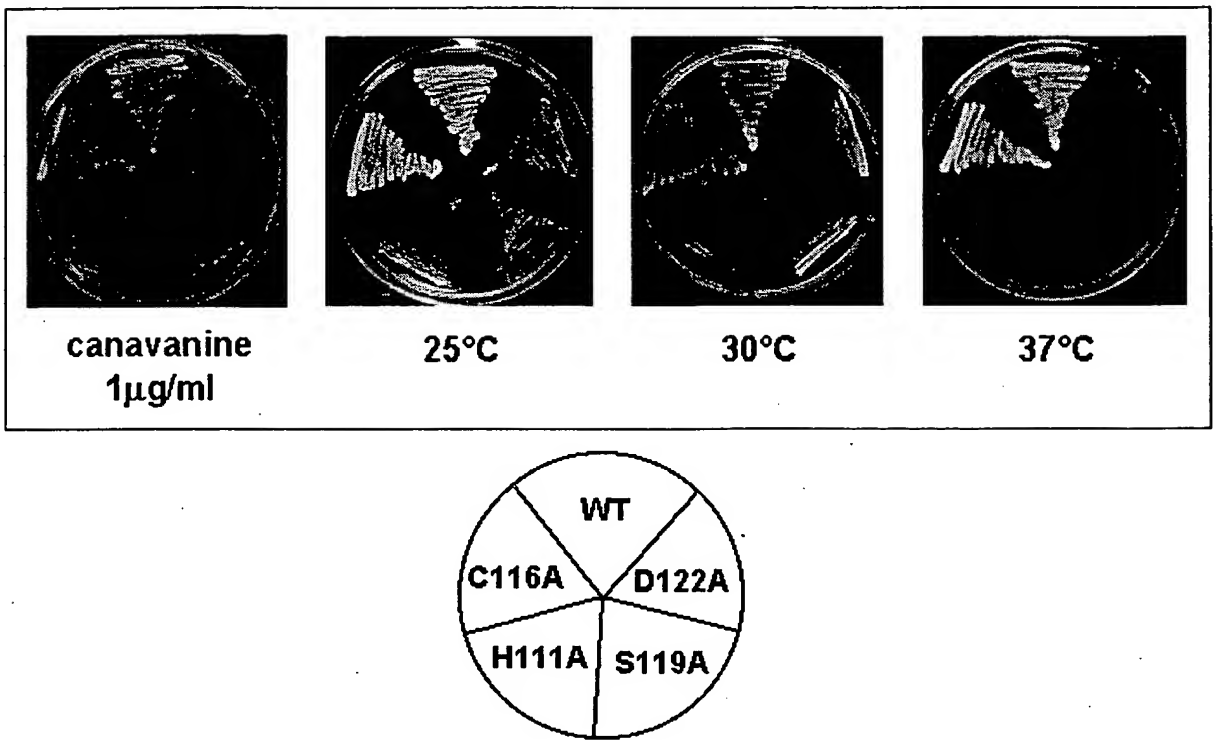


Figure 2
Growth of *rpn11* Mutants under Different Growth Conditions. WT and mutant strains were streaked on YPD at 25, 30 and 37°C. Cells were also plated on complete minimal media containing 1 μg/ml of the amino acid analog canavinine instead of arginine. Plates were photographed after 3–5 days. Three single amino acid substitutions in the MPN+ motif of Rpn11 were studied: his111ala, Ser119ala, and asp122ala. For comparison, a substitution of a highly conserved cysteine residue (cys116ala) that is not part of the MPN+ motif (see Fig. 1) was included as well. At 25°C, all MPN+ mutations are viable but slow growing. Hiss111 is extremely temperature sensitive, showing no appreciable growth even when shifted to 30°C, or in the presence of even 1 μg/ml of the amino acid analog canavinine. Asp122 and Ser119 substitutions show lethality under elevated temperature (37°C) or exposure to canavanine. In comparison, Cys116 substitutions show no growth defects under these conditions.

It appears that the MPN+ motif defines the role of Rpn11 in the proteasome but that the conserved Cys116 residue is not critical for proteasome function. The steady state levels of polyubiquitin-protein conjugates are influenced by the rate of ubiquitination on the one hand, and by rates of deubiquitination or proteasome proteolysis on the other. Accumulation of polyubiquitinated proteins in *rpn11* mutants could therefore be due to a slowdown in either proteasome associated deubiquitination or proteasome-dependent proteolysis.

Stabilization of short-lived proteasome substrate

In order to test whether the accumulation of polyubiquitinated conjugates is directly correlated with a defect in proteasome function, we checked whether mutations in the MPN+ motif of Rpn11 bring about stabilization of proteasome substrates. The steady state levels of known short-lived proteins were measured in WT and in a representative *rpn11* mutant strain. In order to estimate the generality of the effect, two different substrates were used: a protein that is ubiquitinated by enzymes of the UFD ubiquitination pathway [27], and a protein that is ubiquitinated by enzymes of the N-end rule pathway [28]. WT Cells, or those harboring the S119A substitution in the MPN+ motif of Rpn11 were transformed with plasmids expressing Arg-β-galactosidase (an N-end rule substrate) or Ub-pro-β-galactosidase (A UFD substrate). Arg-β-gal and Ub-Pro-β-gal are short-lived in wild-type yeast with half-lives of ~2 and ~6 minutes, respectively [29]. Steady state levels of these proteins were compared to the levels of a stable, long-lived protein, Met-β-galactosidase. As expected, WT cells accumulated high levels of the stable Met-βgal but only low levels of the rapidly degraded Arg-β-gal and Ub-Pro-β-gal (Fig. 4). In contrast, the S119A

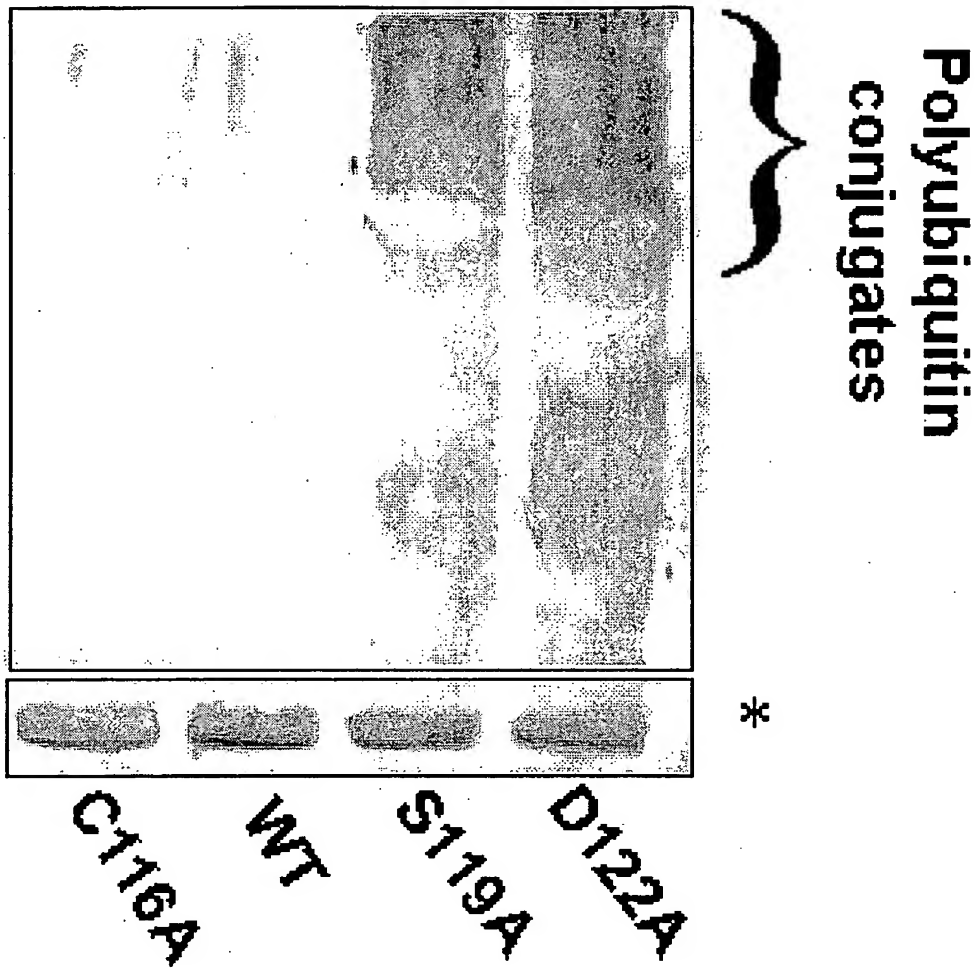


Figure 3
Accumulation of multiubiquitinated proteins in MPN motif mutants. Total cell extracts from WT yeast and from strains containing the MPN+ motif substitutions were separated on an 8% SDS gel and blotted with anti-ubiquitin antibodies. Cells were rapidly lysed in presence of 12% TCA in order to inhibit post lysis enzymatic activity. Accumulation of high MW polyubiquitinated proteins is detected in the Ser119 and Asp122 mutants, but not in WT or in the Cys116 mutants. His111 mutants accumulate polyubiquitinated proteins as well (not shown). A protein band migrating at around 20 kDa that is detected with the anti Ub antibody is used as an internal loading control.

mutation in *rpn11* lead to dramatic stabilization and accumulation of both these short-lived proteins, such that the steady state levels of all three substrates were similar (Fig. 4). Stabilization of these short lived proteins was noted also in the D122A mutant (not shown). From these results we conclude that the MPN+ motif of Rpn11 is essential for proper proteolysis of ubiquitinated substrates by the proteasome. The importance of Rpn11 is independent of the ubiquitination pathway.

The possibility that these deficiencies in proteasome dependent proteolysis were caused by improper incorporation of mutated Rpn11 into the lid, or that proteasome structure was hampered in *rpn11* mutants was addressed

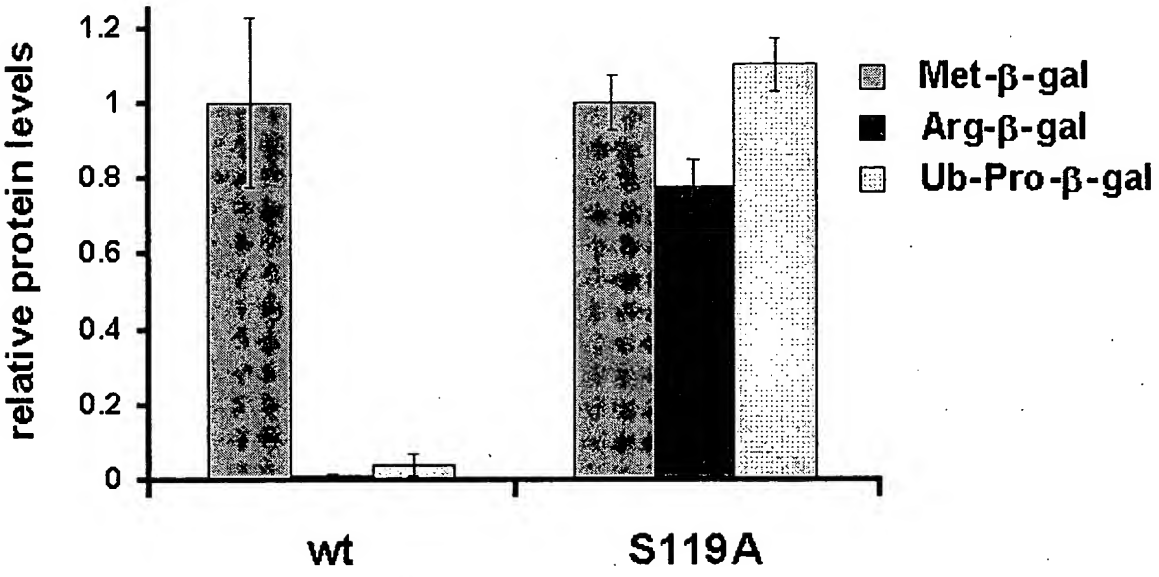


Figure 4
Stabilization of short-lived proteasome substrates. WT and the S119A mutant strains containing the Ub-Met-, Ub-Arg-, and Ub-Pro-βgal constructs on multi-copy plasmids under the GAL promotor were tested for LacZ activity after galactose-induction. LacZ activity is indicative of steady-state levels of the reporter protein. WT cells accumulate high levels of the stable Met-βgal, but rapidly degrade Arg-βgal and UB-Pro-βgal (left panel), whereas the MPN+ mutation shows dramatic stabilization and accumulation of both short-lived fusion proteins (right panel).

by native gel electrophoresis. Cell extract from WT and rpn11 mutants was resolved by nondenaturing PAGE and no gross structural changes were observed (Fig. 5). Overall levels and proteolytic intensities of proteasomes from MPN+ mutants were indistinguishable from WT, indicating that the substitutions do not alter the structure of peptidase function of the proteasome. In addition, we found no evidence for natural abundance WT Rpn11 outside of the proteasome (data not shown), indicating that the phenotypes associated with mutated rpn11 are unlikely to be due to unincorporated protein. Since peptidase activity of mutant proteasomes was similar to WT (Fig. 5), the effects of *rpn11* mutations is most likely due to a defect in proteolysis of ubiquitinated substrates (Fig. 4).

Discussion

We have identified an arrangement of five residues, which are perfectly conserved in a subclass of MPN domains, while none of them shows appreciable conservation outside of this subclass. The highly correlated conservation of these residues suggests that they participate in a common structural element and/or a common function that is critical for the proteins in which they are found. This idea is corroborated by the polar nature of the conserved MPN+ residues: glutamate, histidines, serine and aspartate are all amino acids frequently found in the active site of enzymes or as the coordinating ligands in metal-binding proteins. Certainly, these two possibilities are not mutually exclusive. Several classes of enzymes, particularly metal containing hydrolases and proteases, harbor bound metal ions such as Zn^{2+} as part of their catalytic center [30]. However, while the motif does bear some resemblance to that found in metalloproteases, the specific organization of E-HxH-S-D residues does not correspond to the metal ligands in any of the known Zinc proteases [30], thus positive identification of Rpn11 as a metalloprotease awaits definite proof.

The proteasome complex from a number of sources is known to contain a ubiquitin hydrolyzing activity [31–39], while the CSN complex is known to cause the hydrolytic removal of the Nedd8/Rub1 ubiquitin-like molecule from the cullin subunit of the SCF ubiquitin ligase (E3) complex [40–42]. Thus, the MPN+ containing subunits of

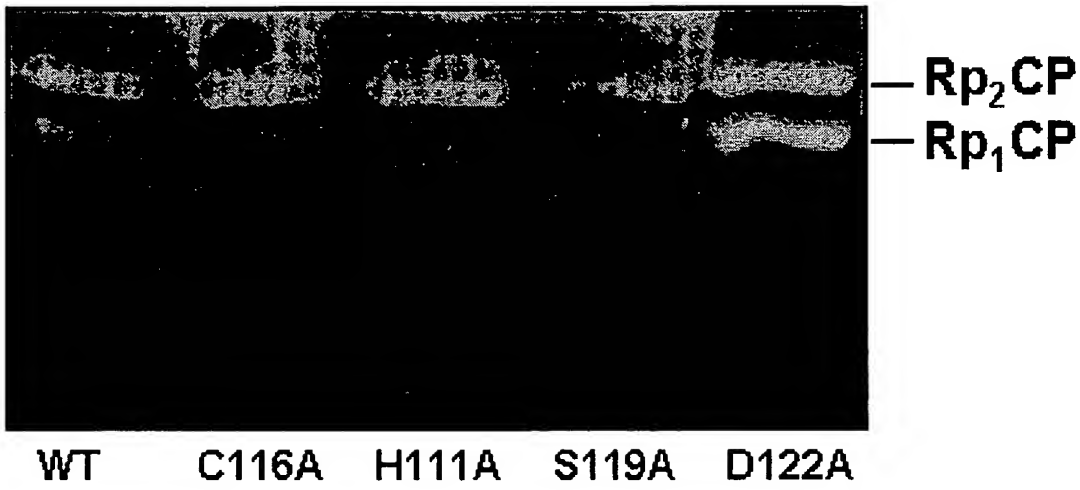


Figure 5
Migration pattern of proteasome from WT and *rpn11* mutants by non-denaturing PAGE. Cell extracts were prepared from Logarithmically growing WT or the *rpn11* MPN+ mutants that were brought to an identical OD600 level. Extracts were clarified by centrifugation, and identical amounts of total protein were separated on native gel. The gel was then incubated for 10 minutes with the fluorescent peptide LLVY-AMC at 30°C, and photographed under UV light (380/440 nm). Fluorescent bands indicate migration of Doubly capped and Singly capped 26S proteasome forms [14]. There is no noticeable change in migration pattern or overall levels of proteasomes from the different rpn11 mutants, indicating that mutated Rpn11 is properly incorporated into the lid and no gross structural changes in proteasome composition or amounts are due to the mutations.

the proteasome lid (Rpn11) and the COP9 signalosome (Csn5) would be prime candidates for such a hydrolytic function. The architecturally related eIF3 complex, for which no such enzymatic activity has been described, is conspicuously devoid of MPN+ proteins. That the CSN from yeast lacks the plain MPN protein (Csn6) retaining only the MPN+ subunit (Csn5), while the plain MPN proteins found in eIF3 from eukaryotes appear to be missing altogether in the yeast complex, emphasizes that the MPN+ residues are likely to be the catalytic residues, with plain MPN subunit playing a redundant structural role in complexes in which they are found. Even though purified recombinant Rpn11 does not appear to exhibit DUB capabilities (data not shown), it is possible that once incorporated into their respective complexes, Rpn11 and Csn5 confer the documented hydrolase activities onto the 19S RP and CSN. In this case, Rpn11 would belong to a unique class of enzymes, as all other known DUBs are cysteine proteases. Of note, the conserved cysteine residue common to both Rpn11 and Csn5, which is not part of the MPN+ motif, is not important for the function of Rpn11.

Single site substitutions in various Rpn11 MPN+ residues exhibit similar phenotypes supporting the identification of MPN+ as a discrete functional motif. Due to the severe growth phenotypes and attenuated ability of the proteasome to proteolyze polyubiquitinated substrates in these mutants, it appears that the MPN+ motif defines the role carried out by Rpn11 in the lid. As we show, these defects arise from an intrinsic activity of Rpn11 within the context of the proteasome, and not due to a gross structural effect upon incorporation of mutated Rpn11. As mentioned above, the lid, where Rpn11 is situated, is critical for proteolysis of polyubiquitinated substrates. So far, and somewhat surprisingly, all ubiquitin binding activity has been mapped to the Base of the 19S RP. Two subunits in the base can interact directly with ubiquitin chains, Rpt5 and Rpn10 [5,7,43,44]. A number of proteins, such as Rad23 and Dsk2, can also bind ubiquitin and interact with the proteasome, presumably with the base, thus they are thought to serve as shuttles of polyubiquitinated substrates to the proteasome [45,46]. It is possible that the lid rather than bind ubiquitin, serves to cleave or trim polyubiquitin chains once attached to the Base.

While this manuscript was under review, two independent papers substantiated our findings by characterizing a novel deubiquitinating activity in the proteasome, and showing that the MPN+ motif residues of Rpn11 are largely responsible for this activity [47,48]. These independent studies identified DUB activity associated with the 19S RP of the proteasome, which is lacking upon lethal substitutions in Rpn11. Interestingly, all substitution mutations that we studied in the MPN+ motif of Rpn11 are viable. Quite possibly, Rpn11 is not the sole proteasome-associated DUB, and a number of DUBs play partially overlapping functions. For instance, Doa4/Ubp4 interacts weakly and substoichiometrically with the proteasome and may serve to release ubiquitin and regenerate the proteasome for the next catalytic cycle [37]. The Ubiquitin-like domain (UBL) containing deubiquitinating enzyme, USP14, has been found to interact with the proteasome from mammalian sources [38]. Ubp6, the budding yeast homolog of USP14, interacts with the proteasome as well [49], and plays a role in proteasome-associated deubiquitination [39]. It has also been reported that the *Drosophila* DUB p37a [31] and its homologs UCH37 (*H. sapiens*) and Uch2 (*S. pombe*), may be responsible for the polyubiquitin chain editing function associated with purified proteasomes [31,34,35]. However, as budding yeast lacks an obvious ortholog of UCH37/p37a, other DUBs must play a greater role in proteasomes from this organism.

Finding MPN+ motif proteins in prokaryotes will help elucidate the origins of proteasome evolution and the function of Rpn11 in particular. The proteasome probably evolved from self-compartmentalized macromolecular proteases found in prokaryotes. Thus, proteasomal subunits, or proteins with motifs related to proteasomal subunits, are present in archaea and certain eubacteria. These "lidless" prototypes include the 20S CP subunits, and homologs of the base ATPases (Rpt subunits) [50]. However, proteins relating to the ubiquitination process, such as ubiquitin itself, ubiquitin activating conjugating or ligating enzymes, and even lid subunits, are missing from prokaryotes. Identification of a motif from Rpn11 – a protein that is linked to processing of polyubiquitinated proteins – in prokaryotic proteins is an interesting development. It is possible that during evolution, the proteasome recruited an existing enzyme as the lid was forming into a regulatory module of the proteasome; likewise for the analogous CSN complex. Studying the prokaryotic MPN+ motif proteins should aid in elucidating what this motif does. The fact that these proteins are the shortest MPN proteins and correspond to the structural core region of the domain should greatly aid in enzymatic and structural studies, especially in comparison to members of the family such as Rpn11 and Csn5 that are naturally found only incorporated into complexes.

Interestingly, all identified prokaryotic MPN domains, both from archaea and from eubacteria, also contain the MPN+ motif. It thus appears likely that the ancestral MPN domain was of the MPN+ type, and that the extant 'plain' MPN proteins have lost this motif later on. It is possible that the plain MPN proteins play a structural role in complexes that additionally contain a MPN+ subunit. So far all known MPN proteins are incorporated into complexes that also contain PCI proteins [51,52]. Interestingly, several of the prokaryotic organisms harboring MPN+ proteins appear to lack PCI domain proteins and therefore probably could not form large PCI/MPN complexes related to the lid and CSN particles. An interesting case is the tail assembly protein 'K' of the bacteriophage lambda (vtak) and its many homologs from other phages and prophages (Fig. 1). Little is known about the specific function of the protein but none of the other tail assembly factors contain a PCI or MPN domain (K.H unpublished results). Our work suggests that the K-protein plays an enzymatic role in tail assembly rather than a merely structural one, although whether the analogy to Rpn11 is relevant to vtak is still enigmatic.

A similar situation exists for the orphan MPN proteins of eukaryotes. It is noteworthy that complex eukaryotic organisms have far more orphan MPN protein than orphan PCI proteins. However, the known PCI/MPN complex particles have stoichiometries that require more PCI components than MPN components. This apparent paradoxon can be resolved by a number of different but non-exclusive assumptions. i) Additional PCI proteins have eluded detection due to high sequence divergence allowing for more than three PCI/MPN complexes to exist, ii) alternative MPN proteins can interact with existing PCI complexes, iii) some MPN proteins have (enzymatic) functions outside of PCI/MPN complexes. Currently, there is no data available supporting the first two possibilities, although there is some promiscuity in the PCI protein interactions [52,53]. The third possibility is likely to be true in prokaryotes and could also explain why all eukaryotic orphan MPN proteins belong to the MPN+ class. This could also explain how supra-stochiometric amounts of Rpn11 exhibit dominant phenotypes; possibly it can function outside of the proteasome as well.

Conclusions

The MPN+ motif is abundant in certain MPN-domain proteins, including newly identified proteins of eukaryotes, bacteria and archaea thought to act outside of the traditional large PCI/MPN complexes. The putative catalytic nature of the MPN+ motif makes it a good candidate for a pivotal enzymatic function, possibly a proteasome-associated deubiquitinating activity and a CSN-associated Nedd8/Rub1-removing activity. The importance of the MPN+ motif for the efficient function of the proteasome

component Rpn11 is compatible with this idea, though the fact that none of the single amino acid substitutions are lethal indicates that either the function of Rpn11 is not strictly essential for proteasome function, or more likely, it is partially redundant with other subunits.

Methods

Bioinformatics

All database searches were performed with a nonredundant data set constructed from current releases of SwissProt, TrEMBL, and GenPept [54,55]. Generalized profile [23] construction and searches were run locally using the *pftools* package, version 2.1. (program available from the URL [ftp://ftp.isrec.isb-sib.ch/sib-isrec/pftools/]). Profiles were constructed using the BLOSUM45 substitution matrix [56] and default penalties of 2.1 for gap opening and 0.2 for gap extension. The statistical significance of profile matches was derived from the analysis of the score distribution of a randomized database as described [24]. Database randomization was performed by individually inverting each protein sequence, using SwissProt 34 as the data source.

Single amino acid substitutions

Haploid yeast strain with a chromosomal knock out of *rpn11* was constructed, in which a single copy URA-marked plasmid expressing the RPN11 gene complements the chromosomal knock out (MY71). The heterozygote *RPN11/rpn11Δ* diploid was purchased from EUROSCAF and transformed with a single copy CEN plasmid with *URA3* selection (ycplac33) expressing *RPN11* from its own promotor (M82). Growth of MY71 was identical to the isogenic WT strain from EUROSCARF. Plasmids expressing the single site substitutions in Rpn11 were generated using PCR site directed mutagenesis on a similar CEN plasmid with the LEU2 marker for selection (ycplac111). In this manner the following plasmids were constructed *RPN11* (M134), *rpn11-C116A* (M134), *rpn11-H111A* (M143), *rpn11-S119A* (M144), *rpn11-D122A* (M145). Those plasmids were then transformed into the above yeast strains, and the *URA3*-marked WT rescue plasmid was then forced out of the cells by the presence of 5'FOA in the medium. FOA shuffling was done at 25°C as most rpn11 mutants were severely temperature sensitive. In this manner we got viable yeast *rpn11* mutant strains.

Phenotypes and mutant characterization

Single colonies grown on YPD at 25°C were streaked onto YPD and shifted to various temperatures. Plates were photographed after 3-5 days. For canavinine sensitivity, plates containing complete minimal media containing 1 mg/ml canavinine in replace of arginine were used and growth measured at 25°C.

Stabilization of known proteasome substrates

Steady-state levels of the Met-, Arg-, and Ub-Pro-βgal fusion proteins were measured by testing LacZ activity [27]. Wild type and rpn11 cells harboring multi-copy URA3-marked plasmids containing each construct were grown to late log in minimal media containing raffinose for carbon source, and induced with 2% Galactose for 4 hrs. The cells were then lysed and LacZ activity upon introduction of the substrate ONPG was calculated when taking into account the time, total protein concentration and amount of product obtained (measured by 405 nm absorbance).

Non-denaturing PAGE and peptidase activity detection

Yeast cells were lysed in buffer A (10% glycerol, 50 mM tris7.4, 1 mM ATP, 1 mM $MgCl_2$, 1 mM EDTA) using glass beads, and clarified by centrifugation at 20 × g. Non-denaturing gel was run as previously described [14]. The gel was then incubated for 10 minutes in 10 ml of buffer A and 0.1 mM of the fluorescent peptide LLVY-AMC at 30°C. Under UV light, appearing bands show peptidase activity indicate the migration pattern of the 26S proteasome.

Abbreviations

RP: 19S regulatory particle of the proteasome

CP: 20S core particle of the proteasome

CSN: COP9 signalosome

eIF3: eukaryotic initiation of translation factor 3

Rpn: regulatory particle non-ATPase subunit

Rpt: Regulatory particle triple-A ATPase subunit

Ub: ubiquitin

DUB: deubiquitinating enzyme

β-gal: β-galactosidase

Authors' contributions

Kay Hofmann and Michael Glickman initiated the project and participated in its design. Kay Hofmann performed bioinformatic characterization of the MPN+ motif (fig. 1). Noa Reis and Vered Maytal designed and constructed the MPN+ amino acid substitutions. Vered Maytal carried out all characterization of Rpn11 mutants shown in Figures 2,3,4,5. All authors read and approved the manuscript.

Acknowledgments

We thank Allen Taylor for anti-Ub antibodies. This work was supported by the German Israel foundation for scientific research (GIF), the Israel Science Foundation (ISF), The Israel Cancer Research Foundation (ICRF), a grant for promotion of research at the Technion, a Technion Vice presidential grant, and the Wolfson fund for study of ubiquitin in health and disease.

References

1. Pickart CM: **Mechanisms underlying ubiquitination** *Annu Rev Biochem* 2001, **70**:503-533
2. Glickman MH, Ciechanover A: **The Ubiquitin-proteasome Proteolytic Pathway: Destruction for the sake of construction.** *Physiol Rev* 2002, **82**:373-428
3. Chung CH, Baek SH: **Deubiquitinating enzymes: Their diversity and emerging roles.** *Biochem Biophys Res Commun* 1999, **266**:633-640
4. D'Andrea A, Pellman D: **Deubiquitinating enzymes: A new class of biological regulators.** *Crit Rev Biochem Mol Biol* 1998, **33**:337-332
5. Glickman MH, Rubin DM, Coux O, Wefes I, Pfeifer G, Cjeka Z, Baumeister W, Fried VA, Finley D: **A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9/Signalosome and eIF3.** *Cell* 1998, **94**:615-623
6. Braun BC, Glickman MH, Kraft R, Dahlmann B, Kloetzel PM, Finely D, Schmidt M: **The base of the proteasome regulatory particle exhibits chaperone-like activity.** *Nat Cell Biol* 1999, **1**:221-226
7. Lam YA, Lawson TG, Velayutham M, Zweier JL, Pickart CM: **A proteasomal ATPase subunit recognizes the polyubiquitin degradation signal.** *Nature* 2002, **416**:763-767
8. Groll M, Bajorek M, Koehler A, Moroder L, Rubin D, Huber R, Glickman MH, Finley D: **A gated channel into the core particle of the proteasome.** *Nat Struct Biol* 2000, **7**:1062-1067
9. Koehler A, Cascio P, Legget DS, Woo KM, Goldberg AL, Finley D: **The axial channel of the proteasome core particle is gated by the Rpt2 ATPase and controls both substrate enry and prodcut release.** *Mol Cell* 2001, **7**:1143-1152
10. Aravind L, Ponting CP: **Homologues of 26S proteasome subunits are regulators of transcription and translation.** *Protein Sci* 1998, **7**:1250-1254
11. Hofmann K, Bucher P: **The PCI domain: A common theme in three multi-protein complexes.** *Trends Biochem Sci* 1998, **23**:204-205
12. Fu HY, Reis N, Lee Y, Glickman MH, Vierstra R: **Subunit interaction maps for the regulatory particle of the 26s proteasome and the cop9 signalosome reveal a conserved core structure.** *EMBO J* 2001, **20**:7096-7107
13. Tsuge T, Matsui M, Wei N: **The subunit 1 of the COP9 signalosome suppresses gene expression through its N-terminal domain and incorporates into the complex through the PCI domain.** *J Mol Biol* 2001, **305**:1-9
14. Glickman MH, Rubin DM, Fried VA, Finley D: **The regulatory particle of the *S. cerevisiae* proteasome.** *Mol Cell Biol* 1998, **18**:3149-3162
15. Glickman MH, Maytal V: **Regulating the 26S proteasome.** *In Curr. Top. Microbiol. and Immunol (Edited by: P. Zwickl and W. Baumeister) Springer-Verlag: Germany* 2002, 43-72
16. Rinaldi T, Ricci C, Porro D, Bolotin-Fukuhara M, Frontali L: **A mutation in a novel yeast proteasomal gene produces a cell cycle arrest, overreplication of nuclear and mitochondrial DNA and an altered mitocondrial morphology.** *Mol Biol Cell* 1998, **9**:2917-2931
17. Rinaldi T, Ricordy R, Bolotin-Fukuhara M, Frontali L: **Mitochondrial effects of the pleiotropic proteasomal mutation mpr1/rpn11: uncoupling from cell cycle defects in extragenic revertants.** *Gene* 2002, **286**:43-51
18. Stitzel ML, Durso R, Reese JC: **The proteasome regulates the UV-induced activation of the AP-1-like transcription factor Gcn4.** *Genes Dev* 2001, **15**:128-133
19. Shimanuki M, Saka Y, Yanagida M, Toda T: **A novel essential fission yeast gene pad1(+) positively regulates pap1(+)-dependent transcription and is implicated in the maintenance of chromosome structure.** *J Cell Sci* 1995, **108**:569-579
20. Spataro V, Toda T, Craig R, Seeger M, Dubiel W, Harris AL, Norbury C: **Resistance to diverse drugs and UV light conferred by overexpression of a novel 26S proteasome subunit.** *J Biol Chem* 1997, **272**:30470-30475
21. Nabhan JF, Hamdan FF, Ribeiro P: **A *Schistosoma mansoni* Pad1 homologue stabilizes c-Jun.** *Mol Biochem Parasitol* 2002, **121**:163-172
22. Tabb MM, Tongaonkar P, Vu L, Nomura M: **Evidence for separable functions of Srp1p, the yeast homolog of importin alpha (Karyopherin alpha): role for Srp1p and Sts1p in protein degradation.** *Mol Cell Biol* 2000, **20**:6062-6073
23. Bucher P, Karplus K, Moeri N, Hofmann K: **A flexible motif search technique based on generalized profiles.** *Comput Chem* 1996, **20**:3-23
24. Hofmann K: **Sensitive protein comparisons with profiles and hidden Markov models.** *Brief Bioinform* 2000, **1**:167-178
25. Tanaka N, Kaneko K, Asao H, Kasai H, Endo Y, Fujita T, Takeshita T, Sugamura K: **Possible involvement of a novel STAM-associated molecule "AMSH" in intracellular signal transduction mediated by cytokines.** *J Biol Chem* 1999, **274**:19129-19135
26. Rubin DM, Glickman MH, Larsen CN, Dhruvakumar S, Finley D: **Active site mutants in the six regulatory particle ATPases reveal multiple roles for ATP in the proteasome.** *EMBO J* 1998, **17**:4909-4919
27. Johnson ES, Ma PC, Ota IM, Varshavsky A: **A proteolytic pathway that recognizes ubiquitin as a degradation signal.** *J Biol Chem* 1995, **270**:17442-17456
28. Bachmair A, Varshavsky A: **The degradation signal in a short-lived protein.** *Cell* 1989, **56**:1019-1032
29. Johnson ES, Bartel B, Seufert W, Varshavsky A: **Ubiquitin as a degradation signal.** *EMBO J* 1992, **11**:497-505
30. Coleman JE: **Zinc enzymes.** *Curr Opin Chem Biol* 1998, **2**:222-234
31. Hoelzl H, Kapelari B, Kellermann J, Seemuller E, Sumegi M, Udvardy A, Medalia O, Sperling J, Muller S, Engel A, Baumeister W: **The regulatory complex of Drosophila melanogaster 26S proteasomes. Subunit composition and localization of a deubiquitylating enzyme.** *J Cell Biol* 2000, **150**:119-130
32. Layfield R, Franklin K, Landon M, Walker G, Wang P, Ramage R, Brown A, Love S, Urquhart K, Muir T, Baker R, Mayer RJ: **Chemically synthesized ubiquitin extension proteins detect distinct catalytic capacities of deubiquitinating enzymes.** *Anal Biochem* 1999, **274**:40-49
33. Lam YA, Xu W, DeMartino GN, Cohen RE: **Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome.** *Nature* 1997, **385**:737-740
34. Lam YA, DeMartino GN, Pickart CM, Cohen RE: **Specificity of the ubiquitin isopeptidase in the PA700 regulatory complex of the 26S proteasome.** *J Biol Chem* 1997, **272**:28438-28446
35. Li T, Naqvi NI, Yang H, Teo TS: **Identification of a 26S proteasome-associated UCH in fission yeast.** *Biochem Biophys Res Commun* 2000, **272**:270-275
36. Eytan E, Armon T, Heller H, Beck S, Hershko A: **Ubiquitin C-terminal hydrolase activity associated with the 26 S protease complex.** *J Biol Chem* 1993, **268**:4668-4674
37. Papa FR, Amerik AY, Hochstrasser M: **Interaction of the Doa4 deubiquitinating enzyme with the yeast 26S proteasome.** *Mol Biol Cell* 1999, **10**:741-756
38. Borodovsky A, Kessler BM, Casagrande R, Overkleeft HS, Wilkinson KD, Ploegh HL: **A novel active site-directed probe specific for deubiquitylating enzymes reveals proteasome association of USP14.** *EMBO J* 2001, **20**:5187-5196
39. Legget DS, Hanna J, Borodovsky A, Crossas B, Schmidt M, Baker RT, Walz T, Ploegh HL, Finley D: **Multiple associated proteins regulate proteasome structure and function.** *Mol Cell* 2002
40. Lyapina S, Cope G, Shevchenko A, Serino G, Tsuge T, Zhou C, Wolf DA, Wei N, Shevchenko A, Deshaies RJ: **Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome.** *Science* 2001, **292**:1382-1385
41. Mundt KE, C L, Carr AM: **Deletion Mutants in COP9/Signalosome Subunits in Fission Yeast Schizosaccharomyces pombe Display Distinct Phenotypes.** *Mol Biol Cell* 2002, **13**:493-502
42. Zhou C, Seibert V, Geyer R, Rhee E, Lyapina S, Cope G, Deshaies RJ, Wolf DA: **The fission yeast COP9/signalosome is involved in cullin modification by ubiquitin-related Ned8p.** *BMC Biochem* 2001, **2**:7
43. van Nocker S, Sadis S, Rubin DM, Glickman M, Fu H, Coux O, Wefes I, Finley D, Vierstra RD: **The multiubiquitin chain binding protein Mcb1 is a component of the 26S proteasome in *Saccharomyces cerevisiae* and plays a nonessential, substrate-specific role in protein turnover.** *Mol Cell Biol* 1996, **11**:6020-6028
44. Deveraux Q, Ustrell V, Pickart C, Rechsteiner M: **A 26S subunit that binds ubiquitin conjugates.** *J Biol Chem* 1994, **269**:7059-7061
45. Chen L, Madura K: **Rad23 promotes the targeting of proteolytic substrates to the proteasome.** *Mol Cell Biol* 2002, **22**:4902-4913

46. Funakoshi M, Sasaki T, Nishimoto T, Kobayashi H: **Budding yeast Dsk2p is a polyubiquitin-binding protein that can interact with the proteasome.** *Proc Natl Acad Sci USA* 2002, **99**:745-750
47. Yao T, Cohen RE: **A cryptic protease couples deubiquitination and degradation by the proteasome.** *Nature* 2002
48. Verma R, Aravind L, Oania R, McDonald WH, Yates JR III, Koonin EV, Deshaies RJ: **Role of Rpn11 Metalloprotease in Deubiquitination and Degradation by the 26S Proteasome.** *Science* 2002
49. Gavin AC, Bosche M, Krause R, Grandi P, Marzioch M, Bauer A, Schultz J, Rick JM, Michon AM, Cruciat CM, Remor M, Hofert C, Schelder M, Brajenovic M, H R, A M, K K, M H, Dickson D, Rudi T, Gnau V, Bauch A, Bastuck S, Huhse B, Leutwein C, Heurtier MA, Copley RR, Edelmann A, Querfurth E, Rybin V, Drewes G, Raida M, Bouwmeester T, Bork P, Seraphin B, Kuster B, Neubauer G, Superti-Furga G: **Functional organization of the yeast proteome by systematic analysis of protein complexes.** *Nature* 2002, **415**:141-147
50. Volker C, Lupas A: **Molecular Evolution of Proteasomes.** *In Curr. Top. Microbiol. and Immunol (Edited by: P. Zwickl and W. Baumeister) Springer-Verlag: Berlin Heidelberg* 2002, 1-22
51. Chamovitz DA, Segal D: **JAB1/CSN5 and the COP9 signalosome. A complex situation.** *EMBO Rep* 2001, **2**:96-101
52. Kim TH, Hofmann K, von Armin AG, Chamovitz DA: **PCI complexes: pretty complex interactions in diverse signaling pathways.** *Trends Plant Sci* 2001, **6**:379-386
53. Karniol B, Yahalom T, Kwok S, Tsuge T, Matsui M, Deng XW, Chamovitz DA: **The *Arabidopsis* homologue of an eIF3 complex subunit associates with the COP9 complex.** *FEBS Lett* 1998, **439**:173-179
54. Bairoch A, Apweiler R: **The Swiss-Prot Protein Sequence Data Bank and Its Supplement Trembl.** *Nucleic Acids Research* 1997, **25**:31-36
55. Benton D: **Recent changes in the GenBank On-line Service.** *Nucleic Acids Research* 1990, **18**:1517-20
56. Henikoff S, Henikoff JG: **Amino acid substitution matrices from protein blocks.** *Proceedings of the National Academy of Sciences of the United States of America* 1992, **89**:10915-9

Publish with **BioMed** Central and every scientist can read your work free of charge

"BioMedcentral will be the most significant development for disseminating the results of biomedical research in our lifetime."

Paul Nurse, Director-General, Imperial Cancer Research Fund

Publish with **BMC** and your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours - you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/manuscript/

editorial@biomedcentral.com

of ubiquitin conjugates from proteasome substrates (*32*). There exist other Csn5/Rpn11 homologs in eukaryotes (Fig. 1) and, by extension, we propose the "JAMMIN" hypothesis, which posits that eukaryotic JAMM proteins are isopeptidases that deconjugate Nedd8 or other ubiquitin-like proteins.

Drosophila sustained by Csn5 carrying a JAMM domain mutation arrest development as larvae with abnormalities in photoreceptor differentiation, suggesting that at least two functions associated with Csn5—viability and photoreceptor differentiation—require its JAMM-dependent isopeptidase activity. Given that Csn5 has been implicated in c-jun signaling (*12*), p27 turnover (*33*), cytokine signaling (*14*), and growth cone-target interactions (*11*), it will be interesting to see if isopeptidase activity of Csn5 underlies these diverse processes as well.

All neddylated proteins known are members of the cullin family. It is not clear whether CSN isopeptidase acts exclusively upon cullin-Nedd8 conjugates or cleaves other targets. Regardless, given the large number of F-box proteins and the potential for substantial diversity in the substrates for SCF and other cullin-based ubiquitin ligases, CSN deneddylase activity may play an enormous role in cellular regulation.

References and Notes

1. D. Skowyra, K. L. Craig, M. Tyers, S. J. Elledge, J. W. Harper, *Cell* **91**, 209 (1997).
2. R. M. Feldman, C. C. Correll, K. B. Kaplan, R. J. Deshaies, *Cell* **91**, 221 (1997).
3. R. J. Deshaies, *Annu. Rev. Cell Dev. Biol.* **15**, 435 (1999).
4. F. Osaka *et al.*, *EMBO J.* **19**, 3475 (2000).
5. T. Kawakami *et al.*, *EMBO J.* **20**, 4003 (2001).
6. S. Lyapina *et al.*, *Science* **292**, 1382 (2001).
7. C. Schwechheimer *et al.*, *Science* **292**, 1379 (2001).
8. M. Seeger, C. Gordon, W. Dubiel, *Curr. Biol.* **11**, R643 (2001).
9. N. Wei, X. W. Deng, *Trends Genet.* **15**, 98 (1999).
10. S. Freilich *et al.*, *Curr. Biol.* **9**, 1187 (1999).
11. G. S. Suh *et al.*, *Neuron* **33**, 35 (2002).
12. F. X. Claret, M. Hibi, S. Dhut, T. Toda, M. Karin, *Nature* **383**, 453 (1996).
13. R. Kleemann *et al.*, *Nature* **408**, 211 (2000).
14. E. Bianchi *et al.*, *Nature* **404**, 617 (2000).
15. G. Cope, R. Deshaies, unpublished data.
16. C. Zhou *et al.*, *BMC Biochem.* [online] **2**, article #7 (2001). Available at: www.biomedcentral.com/bmcbiochem.
17. S. F. Altschul *et al.*, *Nucleic Acids Res.* **25**, 3389 (1997).
18. L. Aravind, C. P. Ponting, *Protein Sci.* **7**, 1250 (1998).
19. K. E. Mundt *et al.*, *Curr. Biol.* **9**, 1427 (1999).
20. C. P. Ponting, L. Aravind, J. Schultz, P. Bork, E. V. Koonin, *J. Mol. Biol.* **289**, 729 (1999).
21. N. D. Rawlings, A. J. Barrett, *Methods Enzymol.* **248**, 183 (1995).
22. Like JAMM, metal-dependent peptidases often possess two conserved acidic residues: one that acts as a ligand for zinc and a second that acts as a nucleophile to promote attack by water on the peptide backbone. Based on the common occurrence of Glu as a nucleophile and on the predicted secondary structure of the Jab/MPN domain, we propose that the Glu helps polarize a water molecule for hydrolytic attack and the COOH-terminal His and Asp residues serve as three ligands for zinc.
23. N. Wei, X. W. Deng, *Photochem. Photobiol.* **68**, 237 (1998).
24. A. G. Loewy *et al.*, *J. Biol. Chem.* **268**, 9071 (1993).
25. D. R. Holland, A. C. Hausrath, D. Juers, B. W. Matthews, *Protein Sci.* **4**, 1955 (1995).
26. M. Gomez-Ortiz, F. X. Gomis-Ruth, R. Huber, F. X. Aviles, *FEBS Lett.* **400**, 336 (1997).
27. D. Lammer *et al.*, *Genes Dev.* **12**, 914 (1998).
28. D. Liakopoulos, G. Doenges, K. Matuschewski, S. Jentsch, *EMBO J.* **17**, 2208 (1998).
29. A. C. Gavin *et al.*, *Nature* **415**, 141 (2002).
30. V. N. Podust *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 4579 (2000).
31. Null mutant survival up to third instar larvae was context dependent. Although Csn5-null larvae rarely survived to third instar at UCLA, we routinely found that these mutants survived to third instar at CalTech.
32. R. Verma *et al.*, *Science* **298**, 611 (2002).
33. K. Tomoda, Y. Kubota, J. Kato, *Nature* **398**, 160 (1999).
34. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
35. C. Notredame, D. G. Higgins, J. Heringa, *J. Mol. Biol.* **302**, 205 (2000).
36. B. Rost, C. Sander, R. Schneider, *Comput. Appl. Biosci.* **10**, 53 (1994).
37. Materials and Methods are available as supporting material on *Science* Online.

38. We thank D. Wolf, M. Hochstrasser, K. Mundt, and A. Carr for generously providing yeast strains and plasmids, the S. Benzer lab for providing lab equipment and space, and S. Lyapina for psCSN. We thank members of the Deshaies lab for providing helpful insight and discussions. This work was supported by NIH (G.A.C.) and the Howard Hughes Medical Institute.

Supporting Online Material

www.sciencemag.org/cgi/content/full/1075901/DC1
Materials and Methods
Figs. S1 and S2
References and Notes

9 July 2002; accepted 7 August 2002
Published online 15 August 2002;
10.1126/science.1075901
Include this information when citing this paper.

Role of Rpn11 Metalloprotease in Deubiquitination and Degradation by the 26*S* Proteasome

Rati Verma,[1] L. Aravind,[2] Robert Oania,[1] W. Hayes McDonald,[3] John R. Yates III,[3] Eugene V. Koonin,[2] Raymond J. Deshaies[1]*

The 26*S* proteasome mediates degradation of ubiquitin-conjugated proteins. Although ubiquitin is recycled from proteasome substrates, the molecular basis of deubiquitination at the proteasome and its relation to substrate degradation remain unknown. The Rpn11 subunit of the proteasome lid subcomplex contains a highly conserved Jab1/MPN domain–associated metalloisopeptidase (JAMM) motif—$EX_nHXHX_{10}D$. Mutation of the predicted active-site histidines to alanine (*rpn11AXA*) was lethal and stabilized ubiquitin pathway substrates in yeast. $Rpn11^{AXA}$ mutant proteasomes assembled normally but failed to either deubiquitinate or degrade ubiquitinated Sic1 in vitro. Our findings reveal an unexpected coupling between substrate deubiquitination and degradation and suggest a unifying rationale for the presence of the lid in eukaryotic proteasomes.

Proteolysis by the 26*S* proteasome proceeds by binding of the ubiquitinated substrate protein to the 19*S* regulatory particle, followed by its unfolding and translocation into the lumen of the 20*S* core, where it is degraded by the action of the 20*S* peptidases (*1*–*3*). At some point in this process, the ubiquitin targeting signal is detached from the substrate. It is appealing to envision that this deubiquitination is obligatorily coupled to degradation. Such coupling would render the targeting event irreversible, prevent unproductive turnover of ubiquitin, and presumably alleviate steric blockade of the 20*S* core entry portal by the bulky ubiquitin chain, which is linked by isopeptide bonds. When and where substrate deubiquitination takes place, the identity of the deubiquitinating enzyme (DUB), and whether deubiquitination of a substrate is essential for its degradation by the proteasome are unclear (*4*, *5*).

Budding yeast ubiquitinated S-Cdk inhibitor Sic1 (Ub-Sic1) is rapidly degraded by purified 26*S* proteasomes (*3*, *6*) in a reaction that recapitulates physiological requirements for Sic1 proteolysis (*7*, *8*). To investigate whether degradation of Sic1 is normally accompanied by its deubiquitination, we evaluated the fate of Ub-Sic1 after inhibition of 26*S* proteolytic activity. Epoxomicin inhibits the proteasome by covalently binding the catalytically active β subunits of the 20*S* core (*9*). Purified 26*S* proteasomes were preincu-

[1]Department of Biology and Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA 91125, USA. [2]National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD 20894, USA. [3]Department of Cell Biology, The Scripps Research Institute, San Diego, CA 92037, USA.

*To whom correspondence should be addressed. E-mail: deshaies@caltech.edu

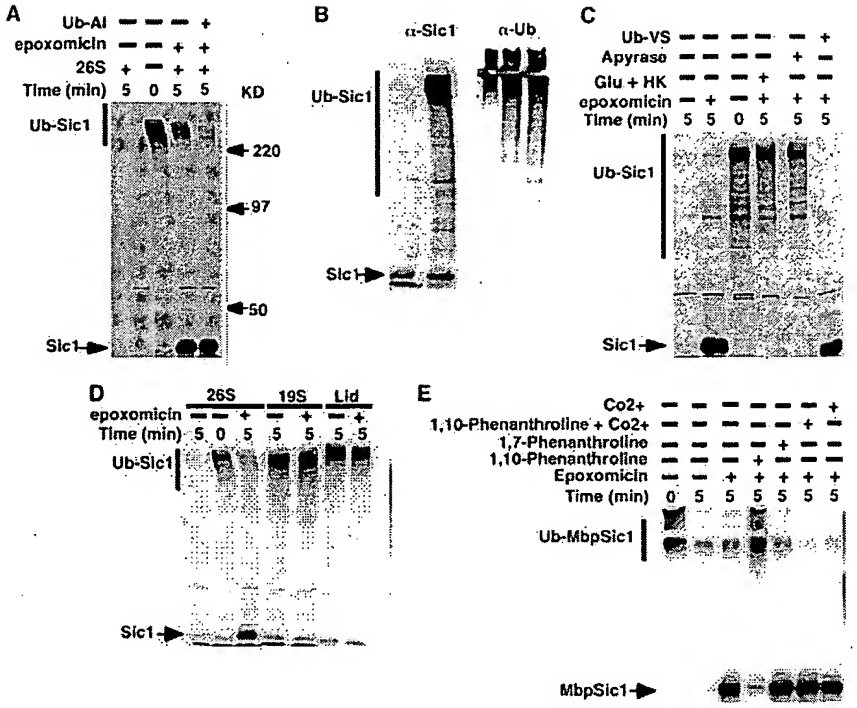
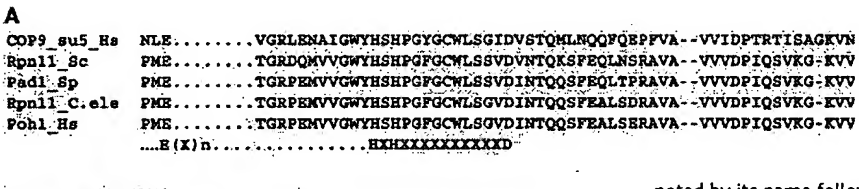


Fig. 1. Characterization of Ub-Sic1 deubiquitination by purified 26*S* proteasomes. (**A**) Ub-Sic1 is deubiquitinated by epoxomicin-treated 26*S* proteasomes in a ubiquitin aldehyde–insensitive manner. Purified proteasomes (100 nM) were incubated with either 1% dimethyl sulfoxide (DMSO) (lanes 1 and 2, mock), or 100 μM epoxomicin in the absence (lane 3) or presence (lane 4) of 2.5 μM ubiquitin aldehyde (Ub-Al) for 30 min at 30°C. The degradation reaction was initiated by the addition of Ub-Sic1 (300 nM) and 1× ATP regenerating system (1× ARS) (*3*), incubated at 30°C for 5 min, and terminated by the addition of SDS sample buffer. Aliquots were resolved by SDS-PAGE (8% polyacrylamide gel), transferred to nitrocellulose, and immunoblotted with polyclonal antibody to Sic1 to monitor degradation. (**B**) Sic1 generated by epoxomicin-treated proteasomes is completely deubiquitinated. An aliquot of the epoxomicin-treated sample from lane 3 in (A) as well as a Ub-Sic1 preparation containing both Ub-Sic1 and unmodified Sic1 were resolved by SDS-PAGE and immunoblotted with antiserum to Sic1 (α-Sic1) (left) or to ubiquitin (α-Ub) (right). The anti-ubiquitin immunoreactivity is primarily derived from autoubiquitinated Cdc34 (*30*), which is not degraded (*31*). (**C**) Deubiquitination of Ub-Sic1 is ATP dependent. Mock- or epoxomicin-treated 26*S* proteasomes were depleted of ATP by 5 min of incubation with apyrase (15 units/ml) or hexokinase (5 units/ml) plus 30 mM glucose (Glu + HK) before incubation with Ub-Sic1. Lane 6 is the same as lane 2, except the DUB inhibitor ubiquitin vinyl sulfone (Ub-VS) (*32*) was included at 2.5 μM. (**D**) Subparticles of the 26*S* proteasome do not efficiently deubiquitinate Ub-Sic1. The 19*S* regulatory particle was isolated as described in (*6*). The lid subparticle of 19*S* was purified from a strain containing *RPN8TEV2MYC9* as described (*23*). ACoomassie blue–stained preparation is shown in Fig. 4C. The three preparations—26*S*, 19*S*, and lid—were incubated in the absence (lanes 1, 2, 4, and 6) or presence (lanes 3, 5, and 7) of epoxomicin and assayed for degradation/deubiquitination of Ub-Sic1 as described in (A). (**E**) Inhibition of deubiquitination by the metal chelator 1,10-phenanthroline. 26*S* proteasomes were preincubated at 30°C with 1% DMSO and 1% MeOH (lanes 1 and 2) or 100 μM epoxomicin and 1% MeOH (lane 3) containing in addition 1 mM 1,10-phenanthroline (lane 4), 1 mM 1,7-phenanthroline (lane 5), or 1 mM 1,10-phenanthroline premixed with 2 mM $CoCl_2$ (lane 6) or 2 mM $CoCl_2$ (lane 7). The degradation reaction was initiated by the addition of Ub-MbpSic1.



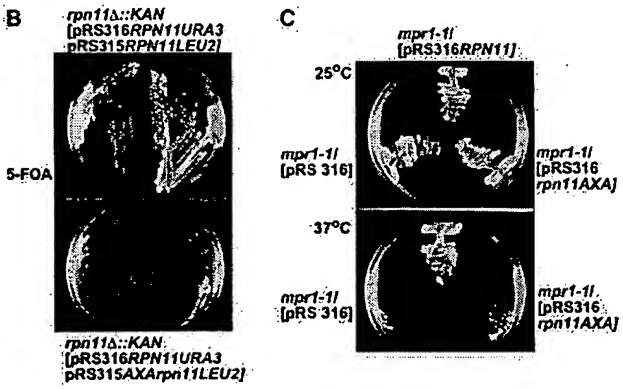


Fig. 2. Rpn11, an intrinsic subunit of the lid subcomplex of the 19*S* regulatory particle, contains a conserved, predicted metalloprotease motif (JAMM) that is critical for viability. (**A**) Sequence alignment of *RPN11* orthologs with human CSN5/JAB1 reveals the JAMM motif. The multiple alignment was constructed by using the T-Coffee program (*33*). Each protein is denoted by its name followed by an abbreviated species name. The predicted metal-chelating and catalytic residues are shown as a consensus below the alignment. For a detailed description of the extended JAMM domain consensus based on all *RPN11* orthologs, see fig. S3. (**B**) An intact JAMM motif is critical for yeast cell proliferation. An *rpn11Δ::KAN* strain kept alive by an [*RPN11 URA3*] plasmid (RJD 1922) was transformed with *LEU2*-marked *RPN11* (RJD 1934) or *rpn11AXA* plasmids (RJD 1935) (*23*). Transformants (two representative clones each) were streaked onto 5-fluoroorotic acid (FOA) plates and allowed to grow for 5 days at 25°C. Because 5-FOA is toxic to *URA3* cells, growth is observed only when the *URA3*-marked plasmid is rendered dispensable by the *LEU2* plasmid. (**C**) The *rpn11AXA* mutant cannot complement the *mpr1-1* temperature-sensitive allele of *RPN11*. *mpr1-1* (RJD 1786) was transformed with either empty pRS316 [*URA3*] vector or vector containing wild-type *RPN11* (RJD 1815) or *rpn11AXA* (RJD 1816). Individual transformants were streaked on synthetic minimal (SD)–Ura plates and incubated at 25°C or 37°C for 5 days.

bated with or without epoxomicin, Ub-Sic1 was then added, and degradation was monitored by the loss of Sic1 antigen. In the absence of epoxomicin, Ub-Sic1 was completely degraded (Fig. 1A; fig. S1). Surprisingly, in the presence of epoxomicin, a large fraction of Ub-Sic1 molecules were completely deubiquitinated, as judged by comigration with unmodified Sic1 and failure to cross-react with antibodies to ubiquitin (Fig. 1B).

Conversion of Ub-Sic1 to Sic1 differed from conventional thiol protease–mediated deubiquitination in four respects: (i) it was insensitive to the classic DUB inhibitors ubiquitin aldehyde (Ub-Al) and ubiquitin vinyl sulfone (Ub-VS) (Fig. 1, A and C); (ii) it required adenosine triphosphate (ATP) (Fig.

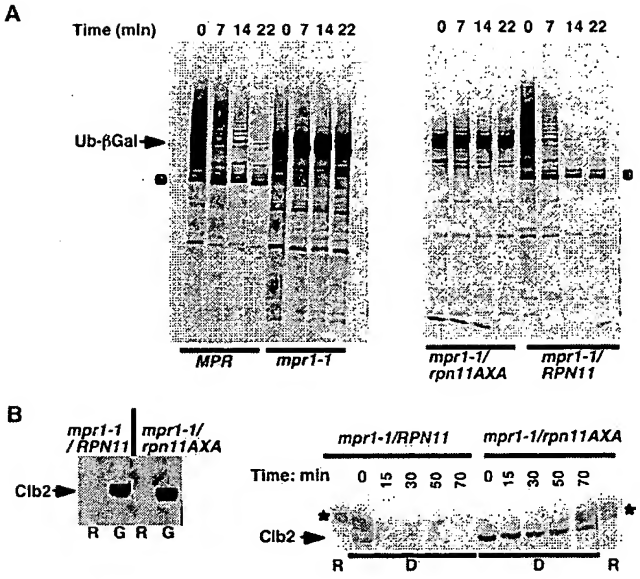


Fig. 3. An intact JAMM motif is required for degradation of ubiquitin–proteasome pathway substrates in vivo. (**A**) Stabilization of a UFD pathway substrate in mutant cells. *RPN11* (RJD1901), *mpr1-1* (RJD 1902), *mpr1-1 RPN11* (RJD 1903), and *mpr1-1 rpn11AXA* (RJD 1904) strains (*23*) containing a reporter plasmid expressing the unstable Ub-V76-Val-eK-B-Gal fusion protein were analyzed by pulse-chase ^{35}S radiolabeling as described in (*6*). Arrow indicates the ^{35}S-labeled substrate protein, and filled circle shows the position of the 90-kD stable breakdown product of the reporter protein. (**B**) Stabilization of mitotic cyclin Clb2 in *mpr1-1 rpn11AXA* cells. Mutant *mpr1-1 RPN11* (RJD 2002) and *mpr1-1/rpn11AXA* (RJD 2004) strains harboring a chromosomally encoded, influenza hemagglutinin antigen (HA)–tagged *GAL-CLB2* expression cassette (*23*) were grown in raffinose medium (R) at 25°C, and Clb2 expression was induced by the addition of 2% galactose (G). To confirm specific expression of Clb2-HA3, we prepared cell lysates by lysing with glass beads and boiling. Aliquots were resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with 12CA5 antibody to HA ascites (left). For evaluation of Clb2-HA3 stability (right), cultures were grown in raffinose (R) medium at 25°C and arrested in G_1 by the addition of α-factor (5 μg/ml) for 2 hours. Clb2 expression was induced with galactose for 1.5 hours, and cultures were shifted to 36°C to inactivate *mpr1-1*. After 1.5 hours, cells were transferred to α-factor–containing dextrose (D) medium at 37°C to extinguish expression of Clb2. Because there is continuous proteolysis of Clb2 in α-factor–arrested cells, and time 0 was collected after washing and resuspension in yeast extract, peptone, and dextrose, the level is much less than that seen in exponential by growing cells (left). Asterisk denotes a nonspecific immunoreactive band to HA.

1C); (iii) it required intact 26*S* proteasome and was not sustained by 19*S* regulatory particle or the lid (Fig. 1D); and (iv) it was completely blocked by *o*-phenanthroline (1,10-phenanthroline), but not *m*-phenanthroline (1,7-phenanthroline) or *o*-phenanthroline presaturated with cobalt ions (Fig. 1E). These four properties suggest that a metallopeptidase was responsible for this activity. These properties are reminiscent of an unidentified deubiquitinating activity that copurifies with 26*S* proteasomes from rabbit reticulocyte lysates (*10*).

We had characterized our proteasome preparation by mass spectrometry, which revealed only a single known DUB, Ubp6 (*3*) (table S1). However, Ubp6 was neither necessary nor sufficient for processing Ub-Sic1 (fig. S2). Thus, we reasoned that the DUB activity we observed might reside in a proteasome subunit that harbors a novel ubiquitin isopeptidase activity.

The lid subcomplex of the 19*S* regulatory particle is necessary for ubiquitin-dependent degradation (*11*). The lid subunits share sequence conservation with subunits of the COP9 signalosome (CSN). CSN preparations contain an isopeptidase activity that promotes cleavage of the ubiquitin-like molecule Nedd8 from Nedd8–Cul1 conjugates (*12*). The Csn5/Jab1 subunit of CSN (*13*, *14*) and the Rpn11 subunit of the lid (Fig. 2A) contain a distinct arrangement of two histidines and an aspartate preceded by a conserved but variably spaced glutamate ($EX_nHXHX_{10}D$) (*15*). We refer to this motif as JAMM for Jab1/Pad1/MPN domain metalloenzyme. We propose that the histidines and aspartate bind a zinc ion, which, together with the upstream glutamate, comprise an active site (*14*).

To evaluate the role of the JAMM motif of Rpn11 in the metal ion–dependent deubiquitination shown in Fig. 1E, we mutated the two conserved histidines to alanines (henceforth referred to as the *rpn11AXA* mutant; table S3). Because Rpn11 is an essential protein (*16*), we evaluated the effect of the mutation by plasmid shuffling. A haploid *rpn11Δ leu2 ura3* strain sustained by a [*RPN11 URA3*] plasmid was transformed with *LEU2* plasmids containing either *RPN11* or *rpn11AXA*. Transformants that harbored [*rpn11AXA LEU2*] were unable to survive without the [*RPN11 URA3*] plasmid (Fig. 2B), indicating that an intact Rpn11 JAMM motif was critical. To facilitate further phenotypic characterization of the *AXA* mutant, we used *mpr1-1*, which contains a frameshift in *RPN11* that leads to temperature-sensitive growth and expression of a prematurely terminated $Rpn11^{mpr1-1}$ protein (285 versus 306 amino acids for wild type) (*16*). Plasmid-borne *RPN11* but not *rpn11AXA* complemented the temperature-sensitive growth of *mpr1-1* (Fig. 2C).

Rpn11 and other subunits of the 19*S* regulatory particle may mediate transcriptional regulation and DNA repair independent of their roles in proteolysis (*17*, *18*). To test whether the inability of *rpn11AXA* to sustain viability might be due to defective protein degradation, we evaluated the stability of an artificial ubiquitin fusion degradation (UFD) pathway substrate (*19*) and the anaphase-promoting complex/cyclosome substrate Clb2 (*20*) in wild-type, *mpr1-1*, *mpr1-1/RPN11*, and *mpr1-1/rpn11AXA* cells. For the latter experiment, cells were arrested in the G_1 phase of the cell cycle with α factor, at which time Clb2 proteolysis proceeds rapidly. *RPN11*, but not *rpn11AXA*, complemented the degradation defects observed for both proteins in *mpr1-1* cells (Fig. 3). Thus, the JAMM motif was essential for substrate proteolysis by the 26*S* proteasome in vivo. Paradoxically, substrates that accumulated in *rpn11AXA* cells were not ubiquitinated. However, when proteolysis is blocked by mutations in proteasome subunits (*21*) or the DUB Doa4 (*22*), substrates accumulate primarily in an unmodified form, presumably because of robust activity of "scavenging" DUBs.

To determine the biochemical basis of the *rpn11AXA* degradation defect, we sought to evaluate the activity of $Rpn11^{AXA}$ proteasomes in vitro. We affinity-purified proteasomes from an *mpr1-1* strain harboring a chromosomal copy of either *RPN11* or *rpn11AXA* (*23*). Immunoblotting with antiserum to Pad1/Rpn11 (*24*) revealed that extracts from *RPN11* and *mpr1-1* contained full-length and truncated Rpn11, respectively, whereas extracts from the *mpr1-1/RPN11* and *mpr1-1/rpn11AXA* strains contained both polypeptides (Fig. 4A). Fortuitously, no truncated $Rpn11^{mpr1-1}$ was present in 26*S* proteasomes prepared from *mpr1-1* strains. This allowed us to prepare point-mutant 26*S* proteasomes that contained $Rpn11^{AXA}$, with no contamination by $Rpn11^{mpr1-1}$.

Native gel electrophoresis followed by Coomassie blue staining and in-gel peptidase assay with a fluorogenic substrate (Fig. 4E) suggested that proteasomes from *mpr1-1* cells were defective for assembly. Indeed, 26*S* proteasomes from the *mpr1-1* strain lacked subunits of the lid subcomplex of the 19*S* regulatory particle as judged by SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 4, B and C) and mass spectrometry (tables S1 and S2). Thus, the COOH terminus of Rpn11 was required either for stabilization of the lid or for association of the base and lid subcom-

plexes in vitro. By contrast, proteasomes recovered from both *mpr1-1/RPN11* and *mpr1-1/rpn11AXA* cells were assembled into functional particles as judged by native gel electrophoresis (Fig. 4E), denaturing SDS-PAGE (Fig. 4B), and immunoblot analysis (Fig. 4D) and contained all 26*S* subunits as determined by mass spectrometry (table S1).

The "isogenic" Rpn11- and Rpn11^{AXA}-containing 26*S* proteasomes were next tested for their ability to deubiquitinate and degrade Ub-Sic1. As expected, Rpn11-containing 26*S* proteasomes degraded Ub-Sic1, and preincubation with epoxomicin blocked degradation, leading to the accumulation of deubiquitinated Sic1 (Fig. 4F). In contrast, the Rpn11^{AXA} 26*S* proteasomes were profoundly defective for both activities. This result suggests a compelling biochemical rationale for the lethality of *rpn11AXA*.

We envision the following sequence of steps in the degradation cycle. After binding of a multiubiquitinated substrate to the 26*S* proteasome, the substrate is unfolded and threaded into the 20*S* core. Concomitantly, the substrate is deubiquitinated by the metalloisopeptidase activity of Rpn11. If Rpn11 activity is blocked, we envision that the tetraubiquitin chain targeting signal, which has a diameter of about 28 Å (as deduced from the crystal structure coordinates in the Protein Data Bank), would sterically block further substrate translocation upon collision with the entry portal of the 20*S* proteasome, which has a diameter comparable to that reported for *Thermoplasma acidophilum* (13 Å) (*25*). Substrate deubiquitination by Rpn11 thus defines a new, key step in protein degradation by 26*S* proteasome. Because deubiquitination of Ub-Sic1 requires ATP and is not sustained by a free lid or 19*S* regulatory particle, we propose that deubiquitination of a substrate is normally coupled tightly to its translocation into the 20*S* core. Although the mechanism underlying this coupling is unknown, we posit that it renders degradation vectorial by preventing deubiquitination until the substrate is irreversibly committed to proteolysis.

Eukaryotic proteasomes are distinguished from bacterial and archaeal ATP-dependent proteases primarily by their dependence on ubiquitin and the presence of the lid subcomplex (*26*). Highly specific targeting of substrates can be mediated by prokaryotic adenosine triphosphatases (ATPases) (*27*, *28*), and Rpt5 ATPase appears to be the proteasomal receptor for ubiquitin chains (*29*). Taken together, these observations raise the question of why eukaryotic proteasomes have a lid. A unifying simplification emerges if one considers that a major—and perhaps only—function of the lid is to serve as a specialized isopeptidase that tightly couples the deubiquitination and degradation of substrates.

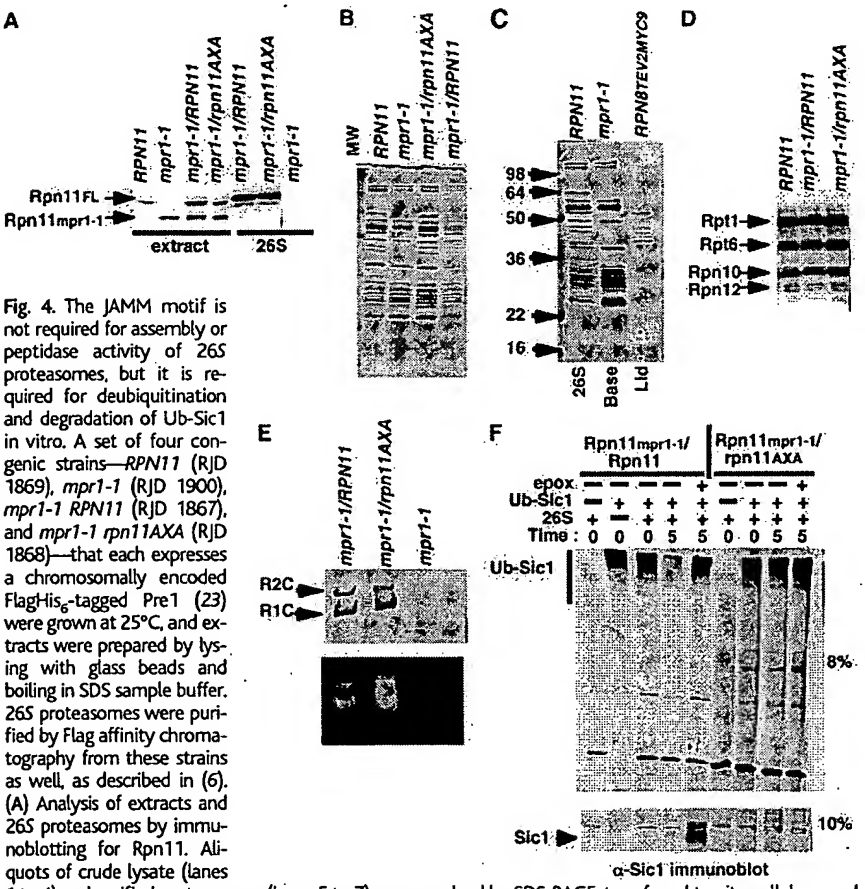


Fig. 4. The JAMM motif is not required for assembly or peptidase activity of 26*S* proteasomes, but it is required for deubiquitination and degradation of Ub-Sic1 in vitro. A set of four congenic strains—*RPN11* (RJD 1869), *mpr1-1* (RJD 1900), *mpr1-1 RPN11* (RJD 1867), and *mpr1-1 rpn11AXA* (RJD 1868)—that each expresses a chromosomally encoded FlagHis$_6$-tagged Pre1 (*23*) were grown at 25°C, and extracts were prepared by lysing with glass beads and boiling in SDS sample buffer. 26*S* proteasomes were purified by Flag affinity chromatography from these strains as well, as described in (*6*). (**A**) Analysis of extracts and 26*S* proteasomes by immunoblotting for Rpn11. Aliquots of crude lysate (lanes 1 to 4) and purified proteasomes (lanes 5 to 7) were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antiserum to Pad1, the *Schizosaccharomyces pombe* Rpn11 ortholog (*24*). (**B** and **C**) *mpr1-1* but not *rpn11AXA* mutation alters polypeptide composition of 26*S* proteasome. Proteasomes isolated from the indicated strains were fractionated by SDS-PAGE and stained with Coomassie blue. The lid subparticle shown in (C) was purified as described in (*23*). (**D**) *rpn11AXA* mutation does not perturb assembly of the 19*S* regulatory particle. Purified 26*S* proteasomes were evaluated by immunoblotting with antibodies specific for the indicated proteasome subunits. (**E**) The *mpr1-1* but not *rpn11AXA* mutation compromises assembly and peptidase activity of 26*S* proteasome. Purified 26*S* proteasomes were resolved by electrophoresis on a 4% nondenaturing polyacrylamide gel and analyzed by Coomassie blue staining (top) or in-gel peptidase activity by incubating with the fluorescent peptidase substrate Suc-LLVY-AMC, as described in (*6*). (**F**) Point-mutant proteasomes with an altered JAMM motif are unable to deubiquitinate and degrade Ub-Sic1 in vitro. 26*S* proteasomes were prepared by anti-Flag affinity chromatography from RJD 1867 (*mpr1-1 RPN11*) and RJD 1868 (*mpr1-1 rpn11AXA*) cells expressing a chromosomally encoded FlagHis$_6$-tagged Pre1. Purified proteasomes were incubated with 2.5 μM ubiquitin aldehyde at 30°C in the presence or absence of 100 μM epoxomicin. Ub-Sic1 and ATP-regenerating system were then added, and degradation was monitored as described in Fig. 1. Lanes 1 and 6 contain the 26*S* proteasome preparations from *mpr1-1 RPN11* and *mpr1-1 rpn11AXA*, respectively, with no Ub-Sic1 added. The same samples were run on an 8% gel (top) as well as a 10% gel (bottom) to better visualize the Ub-Sic1 conjugates and unmodified Sic1.

References and Notes

1. A. Hershko, A. Ciechanover, *Annu. Rev. Biochem.* **67**, 425 (1998).
2. J. Thrower, L. Hoffman, M. Rechsteiner, C. Pickart, *EMBO J.* **19**, 94 (2000).
3. R. Verma, H. McDonald, J. R. Yates III, R. J. Deshaies, *Mol. Cell* **8**, 439 (2001).
4. K. D. Wilkinson, M. Hochstrasser, in *Ubiquitin and the Biology of the Cell*, J. Peters, J. Harris, D. Finley, Eds. (Plenum Press, New York, 1998), pp. 99–125.
5. C. H. Chung, S. H. Baek, *Biochem. Biophys. Res. Commun.* **266**, 633 (1999).
6. R. Verma *et al.*, *Mol. Biol. Cell* **11**, 3425 (2000).
7. E. Schwob, T. Böhm, M. Mendenhall, K. Nasmyth, *Cell* **79**, 233 (1994).
8. R. Verma *et al.*, *Science* **278**, 455 (1997).
9. L. Meng *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 10403 (1999).
10. E. Eytan, T. Armon, H. Heller, S. Beck, A. Hershko, *J. Biol. Chem.* **268**, 4668 (1993).
11. M. Glickman *et al.*, *Cell* **94**, 615 (1998).
12. S. Lyapina *et al.*, *Science* **292**, 1382 (2001).
13. C. P. Ponting, L. Aravind, J. Schultz, P. Bork, E. V. Koonin, *J. Mol. Biol.* **289**, 729 (1999).
14. G. A. Cope *et al.*, *Science* **298**, 608 (2002).
15. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
16. T. Rinaldi, C. Ricci, D. Porro, M. Bolotin-Fukuhara, L. Frontali, *Mol. Biol. Cell* **9**, 2917 (1998).
17. R. C. Conaway, C. S. Brower, J. W. Conaway, *Science* **296**, 1254 (2002).
18. M. L. Stitzel, R. Durso, J. C. Reese, *Genes Dev.* **15**, 128 (2001).
19. E. S. Johnson, B. Bartel, W. Seufert, A. Varshavsky, *EMBO J.* **11**, 497 (1992).

20. A. Amon, S. Irniger, K. Nasmyth, *Cell* **77**, 1037 (1994).
21. W. Seufert, S. Jentsch, *EMBO J.* **11**, 3077 (1992).
22. F. R. Papa, M. Hochstrasser, *Nature* **366**, 313 (1993).
23. Materials and methods are available as supporting material on *Science* Online.
24. M. Shimanuki, Y. Saka, M. Yanagida, T. Toda, *J. Cell Sci.* **108**, 569 (1995).
25. M. Groll *et al.*, *Nature Struct. Biol.* **7**, 1062 (2000).
26. D. Voges, P. Zwickl, W. Baumeister, *Annu. Rev. Biochem.* **68**, 1015 (1999).
27. C. K. Smith, T. A. Baker, R. T. Sauer, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 6678 (1999).
28. R. Verma, R. J. Deshaies, *Cell* **101**, 341 (2000).
29. Y. A. Lam, T. G. Lawson, M. Velayutham, J. L. Zweier, C. M. Pickart, *Nature* **416**, 763 (2002).
30. J. Seol *et al.*, *Genes Dev.* **13**, 1614 (1999).
31. R. Verma, unpublished observations.
32. A. Borodovsky *et al.*, *EMBO J.* **20**, 5187 (2001).
33. C. Notredame, D. G. Higgins, J. Heringa, *J. Mol. Biol.* **302**, 205 (2000).
34. This work was supported by Howard Hughes Medical Institute (R.V. and R.J.D.) and by a grant to University of Washington (W.H.M. and J.R.Y., UOW/RR11823-05-01). We are indebted to C. Crews for epoxomicin, K. Takeda and T. Toda for antiserum to pad1/Rpn11, T. Rinaldi for the MPR and mpr1-1 strains, M. Hochstrasser for ubp6Δ, C. H. Chung for Ubp6 antiserum, G. Alexandru and K. Nasmyth for the GAL-CLB2-HA3 strain, and the Deshaies lab members for critical comments.

Supporting Online Material
www.sciencemag.org/cgi/content/full/1075898/DC1
Materials and Methods
Figs. S1 to S3
Tables S1 to S3

9 July 2002; accepted 7 August 2002
Published online 15 August 2002;
10.1126/science.1075898
Include this information when citing this paper.

Impacts of Soil Faunal Community Composition on Model Grassland Ecosystems

M. A. Bradford,[1]* T. H. Jones,[2] R. D. Bardgett,[3] H. I. J. Black,[4] B. Boag,[5] M. Bonkowski,[6] R. Cook,[7] T. Eggers,[1] A. C. Gange,[8] S. J. Grayston,[9] E. Kandeler,[10] A. E. McCaig,[11]† J. E. Newington,[1] J. I. Prosser,[11] H. Setälä,[12] P. L. Staddon,[13]‡ G. M. Tordoff,[1] D. Tscherko,[10] J. H. Lawton[1,14]

Human impacts, including global change, may alter the composition of soil faunal communities, but consequences for ecosystem functioning are poorly understood. We constructed model grassland systems in the Ecotron controlled environment facility and manipulated soil community composition through assemblages of different animal body sizes. Plant community composition, microbial and root biomass, decomposition rate, and mycorrhizal colonization were all markedly affected. However, two key ecosystem processes, aboveground net primary productivity and net ecosystem productivity, were surprisingly resistant to these changes. We hypothesize that positive and negative faunal-mediated effects in soil communities cancel each other out, causing no net ecosystem effects.

Soil fauna are essential to efficient nutrient cycling, organic matter turnover, and maintenance of soil physical structure, processes that are key determinants of primary production and ecosystem carbon storage (*1–3*). Consequently, there is considerable concern about impacts on ecosystem functioning that might result from shifts in the community composition of soil fauna mediated through global change (*4–6*). Predictions based on theoretical considerations of soil communities are ambivalent. Indeterminate and unexpected impacts are predicted from food web theory (*7*, *8*). Redundancy is also postulated to be common (*9*), with large changes in community composition having minimal effects. Anderson (*10*) argued that net effects may be positive, negative, or zero, depending on the balance between sink and source processes operating at finer scales. Keystone species theory (*11*) and distinct bacterial versus fungal energy channels (*12*, *13*) further cloud the predictions. Therefore, an empirical approach is essential for predicting the impacts of shifts in soil community composition on ecosystem functioning.

Pot experiments with soil, soil organisms, and sometimes an individual plant or plant species have demonstrated the marked potential effects of loss of specific soil fauna and faunal groups on a range of ecosystem processes (*14–16*). However, the validity of extrapolating these studies to the field is questionable given the low species numbers of soil fauna and plants (if present) typically used, the artificiality of the soil, and the limited number of variables measured. What is required is an approach that manipulates the composition of a soil faunal community with a species richness more akin to that in the field, which includes a multispecies plant community and a reconstructed soil profile and measures the response of a suite of interacting variables. To manipulate the soil community in the field, and maintain it over biologically meaningful temporal and spatial scales, is logistically difficult (*17*). Ecological microcosms make such investigations eminently more feasible. We used the Ecotron controlled environment facility (*18*) to test the role of one component of soil community composition—namely, assemblages that differ in animal body sizes—on carbon flux, and microbial and plant community composition and abundance.

We constructed 15 terrestrial microcosms over a period of 7 months (*19*) as analogs of a temperate, acid, sheep-grazed grassland (a habitat that occurs widely across the upland regions of northern Britain). We maintained the microcosms in the Ecotron under constant environmental conditions (*19*) for a further 8.5 months. Soil, plants, fauna, and microorganisms for microcosm construction were collected from the grassland (*19*). Soil fauna were assigned to a functional group according to body width (*20*, *21*) of the adult or, if the adult was not soil dwelling, largest larval stage. Body size provides a good functional classification because it correlates with metabolic rate, generation time, population density, and food size (*22*).

[1]Natural Environment Research Council Centre for Population Biology, Department of Biological Sciences, Imperial College at Silwood Park, Ascot, SL5 7PY, UK. [2]Biodiversity and Ecological Processes Research Group, Cardiff School of Biosciences, Cardiff University, Post Office Box 915, Cardiff, CF10 3TL, UK. [3]Department of Biological Sciences, Institute of Environmental and Natural Sciences, Lancaster University, Lancaster, LA1 4YQ, UK. [4]Centre for Ecology and Hydrology, Merlewood, Grange-over-Sands, LA11 6JU, UK. [5]Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA, UK. [6]Technische Universität Darmstadt, Fachbereich 10, Biologie, D-64287 Darmstadt, Germany. [7]Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, SY23 3EB, UK. [8]School of Biological Sciences, Royal Holloway, University of London, Egham, TW20 0EX, UK. [9]Macaulay Land Use Research Institute, Craigiebuckler, Aberdeen, AB15 8QH, UK. [10]Institute of Soil Science, University of Hohenheim, 70599 Stuttgart, Germany. [11]Department of Molecular and Cell Biology, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen, AB25 2ZD, UK. [12]Department of Ecological and Environmental Sciences, University of Helsinki, Niemenkatu 73, FIN-15140 Lahti, Finland. [13]Department of Biology, University of York, Post Office Box 373, York, YO10 5YW, UK. [14]Natural Environment Research Council, Polaris House, North Star Avenue, Swindon, SN2 1EU, UK.

*To whom correspondence should be addressed. E-mail: m.a.bradford@ic.ac.uk
†Present address: Engineering and Physical Sciences Research Council, Polaris House, North Star Avenue, Swindon, SN2 1ET, UK.
‡Present address: Plant Research Department, Risø National Laboratory, Postbox 49, Roskilde, DK-4000, Denmark.

NCBI PubMed National Library of Medicine NLM

Entrez PubMed Nucleotide Protein Genome Structure OMIM PMC Journals B

Search PubMed for Go Clear

Limits Preview/Index History Clipboard Details

About Entrez

Display Abstract Show: 20 Sort Send to Text

Text Version

Entrez PubMed
Overview
Help | FAQ
Tutorial
New/Noteworthy
E-Utilities

PubMed Services
Journals Database
MeSH Database
Single Citation Matcher
Batch Citation Matcher
Clinical Queries
LinkOut
Cubby

Related Resources
Order Documents
NLM Gateway
TOXNET
Consumer Health
Clinical Alerts
ClinicalTrials.gov
PubMed Central

Privacy Policy

☐ 1: Nature. 1997 Feb 20;385(6618):737-40. Related Articles, Link

Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome.

Lam YA, Xu W, DeMartino GN, Cohen RE.

Department of Biochemistry, University of Iowa, Iowa City 52242, USA.

In eukaryotes, ubiquitin (Ub)-dependent proteolysis is essential for bulk protein turnover as well as diverse processes including cell-cycle control, differentiation, antigen presentation, and the stress response. Generally, multiple ubiquitins are added onto a substrate to form an isopeptide-linked 'polyubiquitin' chain, which targets substrates for degradation by the 26S proteasome. The specificity of Ub-dependent degradation was thought to depend primarily on the selection of targets for ubiquitination, but recently we have reported evidence for a second level of specificity in which (poly) Ub-protein conjugates are partitioned among two fates: degradation of the protein substrate by the 26S proteasome; and disassembly by Ub isopeptidase (s) to regenerate the protein substrate. Potentially, an isopeptidase could influence degradation by 'editing' (poly)Ub-protein conjugates according to the extent of ubiquitination rather than the structure of the ubiquitination target itself. Here we describe a bovine isopeptidase that is well suited to such an editing function because of its unique localization and specificity. This enzyme is an intrinsic subunit of PA700, the 19S regulatory complex of the 26S proteasome. By disassembling the degradation signal from only the distal end of (poly)Ub chains, this isopeptidase can selectively rescue poorly ubiquitinated or slowly degraded Ub-protein conjugates from proteolysis.

PMID: 9034192 [PubMed - indexed for MEDLINE]

Display Abstract Show: 20 Sort Send to Text

Write to the Help Desk
NCBI | NLM | NIH
Department of Health & Human Services
Freedom of Information Act | Disclaimer

Jan 29 2004 15:06: